Metabolic Engineering of Ethanol Production in Thermoanaerobacter mathranii BG1

Yao, Shuo

Publication date:
2009

Document Version
Publisher's PDF, also known as Version of record

Metabolic Engineering of Ethanol Production in *Thermoanaerobacter mathranii*

Risø-PhD-Report

Shuo Yao
Risø-PhD-68(EN)
March 2008

Risø DTU
National Laboratory for Sustainable Energy

Author: Shuo Yao
Title: Metabolic Engineering of Ethanol Production in *Thermoanaerobacter mathranii*
Division: Biosystems Division

Report number:
Risø-PhD-68(EN)

Publication date: March 2008
Published in the Risø-PhD-series in November 2010

ISBN 978-87-550-3858-5

Information Service Department
Risø National Laboratory for Sustainable Energy
Technical University of Denmark
P.O.Box 49
DK-4000 Roskilde
Denmark
Telephone +45 46774005
bibl@risoe.dtu.dk
Fax +45 46774013
www.risoe.dtu.dk

Preface

The work presented in this Ph.D. thesis was conducted at the Bioscience and Technology (BST) Research Group, Department of BioCentrum at the Technical University of Denmark (DTU) from October 1, 2004 to May 2, 2008. The BST Group was merged into the Department of Risø National Laboratory for Sustainable Energy-DTU since January 1, 2008.

Professor, Ph.D. Birgitte K. Ahring was the principle supervisor during the period from October 1, 2004 to February 29, 2008. Thanks to her for introducing me such an interesting topic. The final ending of this Ph.D. study was under the supervision of Professor, Ph.D. Kim Pilegaard from March 1, 2008 to May 2, 2008. I am sincerely grateful to him, who has to come into my area and try his best to help me to finish my Ph.D. study with a big smile.

Ph.D. Marie Just Mikkelsen was performing supervision of my daily experiment work, very special thanks to her for her helpful and stimulating discussions, for her critical and patient reading of the manuscripts and for her wholehearted support to my personal and emotional life during my Ph.D. study. I gratefully acknowledge Slawomir Dabrowski for his helpful suggestions and discussions about the enzyme over-expression and analysis part, for his generous contribution of the expression vectors and strains. Technical support is greatly appreciated from Gitte Hinz Berg and Karin Marie Due.

Professor, Ph.D. Morten Kielland-brandt from DTU Systems Biology, professor, Ph.D. Merja Penttila from VTT of Technical Research Center of Finland, and associate professor, Ph.D. Kjeld Ingvorsen from Aarhus University contributed to the examination of the Ph.D. thesis and its defense. Many thanks for their great efforts to the final ending of this study.

The thesis consists of a short summary, a general introduction that describes the theoretical background of the project and discusses the attained results in relation to the work described in the literature. Results of the project are presented in the separate chapters and published as research papers listed in the appendix.

Finally, the Ph.D. study was financially supported by a Ph.D. scholarship funded by national government grants from the Technical University of Denmark.

Shuo Yao; Lyngby (Denmark); March 2008

Summary

Strain BG1 is a xylanolytic, thermophilic, anaerobic, Gram-positive bacterium originally isolated from an Icelandic hot spring. The strain belongs to the species *Thermoanaerobacter mathranii*. The strain ferments glucose, xylose, arabinose, galactose and mannose simultaneously and produces ethanol, acetate, lactate, CO_2, and H_2 as fermentation end-products. As a potential ethanol producer from lignocellulosic biomass, tailor-made BG1 strain with the metabolism redirected to produce ethanol is needed. Metabolic engineering of *T. mathranii* BG1 is therefore necessary to improve ethanol production.

Strain BG1 contains four alcohol dehydrogenase (ADH) encoding genes. They are *adh*A, *adh*B, *bdh*A and *adh*E encoding primary alcohol dehydrogenase, secondary alcohol dehydrogenase, butanol dehydrogenase and bifunctional alcohol/acetaldehyde dehydrogenase, respectively. The presence in an organism of multiple alcohol dehydrogenases with overlapping specificities makes the determination of the specific role of each ADH difficult. Deletion of each individual *adh* gene in the strain revealed that the *adh*E deficient mutant strain fails to produce ethanol as the fermentation product. The bifunctional alcohol/acetaldehyde dehydrogenase, AdhE, is therefore proposed responsible for ethanol production in *T. mathranii* BG1, by catalyzing sequential NADH-dependent reductions of acetyl-CoA to acetaldehyde and then to ethanol under fermentative conditions. Moreover, AdhE was conditionally expressed from a xylose-induced promoter in a recombinant strain (BG1E1) with a concomitant deletion of a lactate dehydrogenase. Over-expression of AdhE in strain BG1E1 with xylose as a substrate facilitates the production of ethanol at an increased yield.

With a cofactor-dependent ethanol production pathway in *T. mathranii* BG1, it may become crucial to regenerate cofactor to increase the ethanol yield. Feeding the cells with a more reduced carbon source, such as mannitol, was shown to increase ethanol yield beyond that obtained with glucose and xylose. The *ldh* gene coding for lactate dehydrogenase was deleted from strain BG1 to eliminate an NADH oxidation pathway (BG1L1). To further facilitate NADH regeneration used for ethanol formation, a heterologous gene *gldA* encoding an NAD^+ dependent glycerol dehydrogenase (GLDH) was expressed in *T. mathranii* with or without concomitant deletion of a lactate dehydrogenase. With a functional lactate formation pathway, expression of GLDH in a recombinant *T.*

mathranii strain (BG1G2) leads to a significantly decreased ethanol yield accompanied by an increased lactate formation, which was shown to be the preferred route for the regeneration of NAD^+. However, with an inactivated lactate formation pathway, expression of GLDH in another recombinant *T. mathranii* strain (BG1G1) leads to an increased carbon flux channelled towards the production of ethanol over acetate, hence restoring the redox balance. Finally, it was shown that strain BG1G1 acquired the capability to utilize glycerol as an extra carbon source in the presence of xylose, and utilization of the more reuduced substrate glycerol resulted in a higher ethanol yield.

RESUMÉ

Thermoanaerobacter mathranii BG1 er en xylolanolytisk, thermofil, anaerob, Gram-positiv bakteriestamme, der oprindeligt blev isoleret fra en varm kilde på Island. Stammen fermenterer glukose, xylose, arabinose, galactose og mannose samtidigt og producerer derved ethanol, acetat, laktat, hydrogen og kuldioxid som fermenteringsprodukter. Denne stamme har et stort potentiale som produktionsbakterie til omsætning af lignocellulose-holdig biomasse til ethanol. Dog er specifikke modifikationer af bakteriens metabolisme nødvendige før denne proces er rentabel. Modifikationerne indbefatter radikale ændringer af bakteriens metabolisme og er alle nødvendige for at kunne forbedre ethanol produktionen.

I BG1 koder fire gener *adh*A, *adh*B, *bdh*A samt *adh*E for henholdsvis primær alkohol dehydrogenase, sekundær alkohol dehydrogenase, butanol dehydrogenase og bifunktional alkohol/aldehyd dehydrogenase. Forekomsten af flere alkohol dehydrogenaser med overlappende specificiteter gør det vanskeligt at klarlægge specificiteten af hver alkohol dehydrogenase (ADH). Deletion af hvert enkelt *adh*-gen i BG1stammen viste, at den mutant der mangler *adhE* ikke kan producere ethanol som det endelige fermenterings produkt. Den bifunktionelle alkohol/aldehyd dehydrogenase (AdhE) antages derfor at være essentiel for ethanol produktion i *Thermoanaerobacter mathranii* BG1, ved at katalysere de sekventielle NADH-afhængige reduktioner af acetyl-CoA til acetaldehyd og videre til ethanol under fermentative forhold. Ydermere blev *adhE* betinget udtrykt fra en xylose-inducerbar promoter i en stamme (BG1E1), der ikke havde genet for laktat dehydrogenase. Overudtryk af *adhE* i BG1E1 stammen med xylose som substrat førte til et forøget udbytte af ethanol.

Ethanol syntese vejen i *T. mathrani* er co-faktor afhængig. Det kan derfor være af betydning at co-faktorerne kan regenereres, for at ethanol udbyttet kan forøges. Ved at bruge mannitol, en mere reduceret kulstofkilde, vistes det, at ethanol udbyttet blev forøges udover det, der kunne opnås ved at bruge glukose eller xylose som kulstofkilde. For at eliminere NADH oxidations vejen blev genet *ldh*, der koder for laktat dehydrogenase, deleteret fra stammen BG1 (BG1L1). For yderligere at øge NADH regenerationen der er nødvendig for ethanol produktionen, blev et heterologt gen, *gldA,* der koder for en NAD+ afhængig glycerol dehydrogenase (GLDH) udtrykt i *T. mathranii* stammer der enten har eller ikke har laktat dehydrogenase genet. Udtryk af GLDH i en rekombinant *T. mathranii* stamme (BG1G2) med en funktionel laktat syntese vej førte til et signifikant forringet ethanol udbytte, der modsvaredes af en forøget laktat dannelse. Laktat dannelses vistes at være den

foretrukne vej for regeneration af NAD^+. Udtryk af GLDH i en anden rekombinant *T. mathranii* stamme (BG1G1) med et inaktivt laktat syntese vej førte derimod til et forøget flux af kulstof til syntese af ethanol i forhold til syntese af acetat, og med en følgende reetablering af redox balancen. Slutteligt vistes det, at stammen BG1G1 var i stand til at udnytte glycerol sammen med xylose som kulstofkilde. Glycerol udnyttelsen resulterede i forøget ethanol udbytte.

TABLE OF CONTENT

Chapter 1

Introduction

1.1 Background

The relatively high price, and in the long term the limited availability, of fossil fuels has increased concern about future energy and material resources. In addition, the warming of climate, accelerated by carbon dioxide emission from fossil fuels, is alarming. One solution to these challenges could be a transition from traditional oil refineries to biorefineries that would convert renewable organic matter, such as agricultural and municipal solid waste, to energy and value added products [Fernando *et al.* 2006]. One of the major challenges of biorefineries is the generation of transportation fuels. Bioethanol manufactured from renewable resources by microbial fermentation is an attractive alternative because it is carbon dioxide neutral; the amount of CO_2 released in fermentation was originally absorbed from the atmosphere by the growing plants.

Production of ethanol from starch of e.g. wheat, barley or maize by fermentation with the traditional yeast *Saccharomyces cerevisiae* is a well known process. However, the most abundant and cheap renewable raw material for bioethanol production not used for human nutrition is lignocellulose, i.e. plant material consisting of cellulose, hemicellulose and lignin. Today, conversion of cellulose fraction from lignocellulosic biomass into ethanol offers only minor microbiological or technical problems, as opposed to conversion of the hemicellulose fraction [Hagerdal *et al.* 1993]. Few species among the bacteria, yeast and fungi can convert hemicellulose or its monomers (xylose, arabinose, mannose and galactose) into ethanol with a satisfactory yield and productivity [Sommer *et al.* 2004]. Economical analyses have shown that also the hemicellulose fraction needs to be converted to ethanol in order to

obtain an economically feasible lignocellulosic bioconversion process [Hinman *et al.* 1989].

Thermophilic anaerobic bacteria have been examined for their potential as ethanol producers [Sommer *et al.* 2004]. The unique advantage of using these micro-organisms for ethanol production is their ability to metabolize naturally both hexose and pentose sugars found in lignocellulosic biomass [Georgieva *et al.* 2007]. Moreover, there is a large number of potential advantages associated with the production of ethanol at higher temperatures, including high bioconversion rates, low risk of contamination, and energy savings due to cost reduction via cooling, mixing, and distillation [Lynd 1989]. The production of ethanol by thermophilic fermentation using thermophilic anaerobic bacteria offer the potential of direct degradation of cellulose or hemicellulose and direct recovery of ethanol at fermentation temperature under reduced pressure [Burdette *et al.* 2002].

Bioethanol production needs tailor-made organisms in which the metabolism is redirected to utilize both hexose and pentose sugars found in lignocellulosic biomass to produce ethanol. Metabolic engineering of end-product metabolism has been pursued extensively in *Escherichia coli*, resulting in strains of industrial interest that produce high yields of ethanol as well as other products [Desai *et al.* 2004]. Both Gram-positive and thermophilic bacteria have unique advantages compared to Gram-negative bacteria [Dien *et al.* 2003]. However, such metabolic engineering has been pursued only to a limited extent in Gram-positive, mesophilic, obligate anaerobes, and Gram-positive obligate anaerobic thermophiles [Desai *et al.* 2004].

Previous studies on isolation and screening of thermophilic anaerobic bacteria from different natural and man-made systems have identified a strain, *Thermoanaerobacter mathranii* BG1, as a potential ethanol producer from lignocellulosic biomass [Georgieva 2005]. The strain was found to belong to the species of *Thermoanaerobacter mathranii* and to produce mainly ethanol from xylose at neutral pH and 70^{o}C [Mikkelsen and Ahring 2007]. Strain BG1 is originally isolated from an Icelandic hot spring and characterized as a xylanolytic, thermophilic, anaerobic, Gram-positive bacterium. This micro-organism could metabolize glucose, xylose, arabinose, galactose and mannose simultaneously and produce ethanol, acetate, lactic acid, CO_2, and H_2 as fermentation end products [Mikkelsen and Ahring 2007]. Strain BG1 is of interest because of its broad carbohydrate utilization range, high temperature

growth optimum and its potential use for produciton of ethanol from biomass. This organism could grow and produce ethanol from hemicellulose hydrolysate of wheat straw with the same ethanol yield as found for synthetic medium [Georgieva *et al.* 2007]. Genetic modification tools has been developed and used for insertion, deletion or over-expression of the target genes in BG1. The gene encoding lactate dehydrogenase (*ldh*) was deleted, resulting in a significantly improved ethanol producing mutant strain-BG1L1 [Georgieva *et al.* 2007]. In this study, great efforts were made on metabolic engineering of BG1 for improved ethanol production. These genetically modified organisms played a crucial role in our understanding of both genetic and metabolic functions of *T. mathranii* BG1.

1.2 Carbohydrate metabolism in thermophilic bacteria

All of the examined saccharolytic thermophiles employ the Embden Meyerhof pathway for metabolism of glucose [Lamed and Zeikus 1980a, Lamed and Zeikus 1980b] and the presence of non-oxidative pentose phosphate pathway has been established in *Clostridium thermohydrosulfurium* [Ben-Basssat and Zeikus 1981].

Pentose phosphate pathway: D-xylose can be transported into the cell either by active transport or by facilitated diffusion as in *Saccharomyces cerevisiae* [Van Zyl *et al.* 1989]. Bacteria generally employ active transport mechanisms for the uptake of sugars and other nutrients, however, the mechanism is still unknown in *Thermoanaerobacte mathranii*. Inside the cell, D-xylose is isomerized to D-xylulose by the enzyme xylose isomerise (XIM). Phosphorylation of D-xylose to xylulose 5-phosphate marks the beginning of the non-oxidative part of the pentose phosphate pathway [Zubay 1988] (Fig. 1.1). Xylulose 5-phosphate, ribulose 5-phosphate, ribose 5-phosphate, sedoheptulose 7-phosphate and erythrose 4-phosphate can undergo several inter-conversions catalyzed by two enzymes, transaldolase and transketolase, respectively [Zubay 1988]. Transketolase catalyzes the reversible interconversion of four intermediates in the pentose phosphate pathway. Ribose-5-phosphate and xylulose-5 phosphate are converted to glyceraldehydes-3-phosphate and sedoheptulose-7-phosphate. Erythrose 4-phosphate and xylulose 5-phosphate are converted to fructose 6-phosphate and glyceraldehydes 3-phosphate [Zubay 1988]. Transaldolase catalyzes the reversible conversion of sedoheptulose 7-phosphate and glyceraldehydes 3-phosphate to erythrose 4-phosphate and

fructose 6-phosphate. Transaldolase and transketolase therefore catalyze the conversion to the 3-carbon intermediary glyceraldehydes 3-phosphate and the six carbon intermediary fructose 6-phosphate, linking this pathway to the glycolysis [Zubay 1988]. The non-oxidative part of the pentose phosphate pathway and its regulation has not been studied in detail in thermophilic anaerobic bacteria. The oxidative pentose phosphate pathway for generation of NADPH and ribose 5- phosphate for reductive biosynthesis, does not seem to be present in thermophilic anaerobic bacteria [Lamed and Zeikus, 1980a, Jones and Woods 1991]. Ribose 5-phosphate, however, is needed for reductive biosynthesis of histidine, pyridine and pyrimidine [Brock *et al.* 1984]. The generation of this intermediate metabolite can be achieved in the non-oxidative part of the pentose phosphate pathway. D-xylulose-5-phosphate can be epimerized to the D-ribulose-5-phosphate catalyzed by D-ribulose-5-phosphate epimerase and can subsequently be isomerized to ribose-5-phosphate, catalysed by D-ribose 5-phosphate ketol-isomerase.

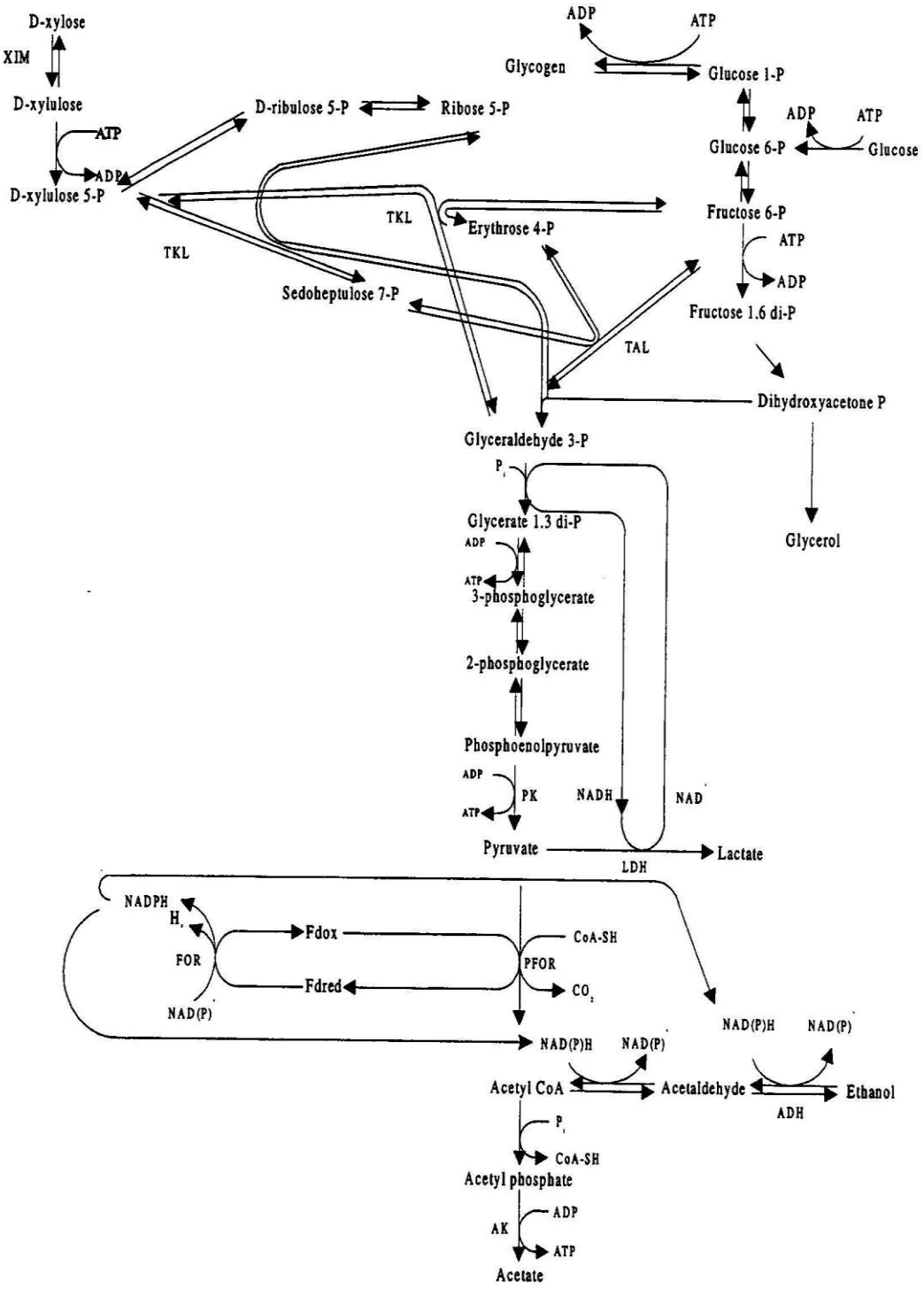

Figure 1.1 Model of anaerobic D-xylose and glucose metabolism in thermophilic anaerobic ethanol producing bacteria. XIM: Xylose isomerase; TKL: Transketolase; TAL: Transaldolase; LDH: Lactate dehydrogenase; FOR: NAD(P)H-ferredoxin oxidoreductase; PFOR: Pyruvate-ferredoxin oxidoreductase; ADH: Alcohol dehydrogenase; AK: Acetate kinase; PK: Pyruvate kinase [Sommer 1998]

Glycolysis pathway: The metabolic flux of the pentose phosphate pathway is then channeled into the upper and middle part of the Embden-Meyerhof pathway as fructose 6-phosphate and glyceraldehydes 3-phosphate, respectively [Zubay 1988]. Fructose 6-phosphate can be converted to glucose 6-phosphate by phosphohexoisomerase and, during anabolism, to glucose-1-phosphate and glycogen (Fig. 1.1). Fructose 6-phosphate and glyceraldehydes 3-phosphate are, during catabolism, converted via pyruvate to the different end fermentation products. This intermediate is a key metabolic branch point, determining the distribution of end-fermentation products. Ferredoxin is present in thermophilic anaerobic bacteria [Lovitt *et al.* 1988, Jones and Woods 1991]. The reduction and oxidation of this molecule is achieved by decarboxylation of pyruvate to acetyl-CoA, production of H_2 or generation of NAD(P)H, respectively. This key metabolic branch point can be controlled by the activities of four oxidoreductases [Jones and Woods 1991]. Pyruvate ferredoxin oxidoreducase catalyzes the conversion of pyruvate to acetyl-CoA and CO_2 with a concomitant reduction of ferredoxin. The re-oxidation of reduced ferredoxin can be catalyzed by three different enzymes. H_2-ferredoxin oxidoreductase (hydrogenase) catalyzes the production of H_2, NADH and NADPH ferredoxin oxidoreductases, catalyzes the production of NADH and NADPH, respectively. The activity of NADH ferredoxin oxidoreductase (oxidation of ferredoxin and concomitant generation of NADH) seems to be low in thermophilic anaerobic bacteria and is strongly inhibited by NADH [Jungermann *et al.* 1973]. The NADPH oxidoreductase is believed to substitute the oxidative part of the pentose phosphate pathway for generation of NADPH for reductive biosynthesis [Jone and Woods 1991]. The generation of NAD^+ and $NADP^+$ by NAD^+ oxidoreductase and $NADP^+$ oxidoreductases, respectively, seem to be absent or very low due to generation of a redox potential by these reactions, unfavourable for generation of reduced ferredoxin [Joneand Wood 1991]. These oxidoreductases and their regulation have not been studied in general in anaerobic thermophiles. The first branch point of the carbohydrate degradation is the conversion of pyruvate to either lactate with the oxidation of NADH or the conversion of pyruvate to acetyl-CoA which is a decarboxylation reaction, resulting in generation of CO_2. The second branch point of the carbohydrate degradation is the conversion of acetyl-CoA to either acetate via acetyl-phosphate with the generation of extra ATP or to ethanol with the

oxidation of NAD(P)H. The formation of the final end-fermentation products and their mutual distribution is determined by the response of the cells to a given metabolic state, their need for energy, metabolites for reductive biosynthesis and reducing equivalents [Zeikus *et al.* 1981].

Acetate and hydrogen production: Pyruvate may be metabolized to acetate with the benefit to the cell that additional ATP is generated by the terminal reaction catalyzed by acetate kinase (AK). The ability to divert acetyl-CoA to regenerate ATP with acetate production is directly linked to the ability of the cell to produce H_2 [Jones and Wood 1991]. To divert all of the acetyl-CoA to acetate, the NADH produced during glycolysis must be reoxidized with the concomitant reduction of ferredoxin and subsequent production of H_2 by hydrogenase. However, the equilibrium of this reaction is unfavourable as it involves a change to a more negative redox potential of H_2 and can only occur if the partial pressure of H_2 is kept very low [Jones and Wood 1991]. In practice, H_2 accumulation also results in inhibition of hydrogenase activity, thus forcing the cell to produce at least some reduced compounds [Mitchell 1998]. *T. mathranii* and related species can maintain the redox balance of the cell by production of other end-fermentation products like ethanol and lactate [Sommer 1998].

Lactate and carbon dioxide production: Conversion of pyruvate to lactate by lactate dehydrogenase (LDH) provides an alternative means of regenerating NAD^+ to maintain the NADH/NAD^+ redox-balance in the glycolysis. Conversion of pyruvate to acetyl-CoA is a decarboxylation reaction, resulting in the generation of CO_2. *T. mathranii* does not have a pyruvate dcarboxylase enzyme, as in yeast, which covers pyruvate to acetaldehyde [Sommer 1998].

Ethanol production: Among the end products of fermentation, ethanol arises from acetyl-CoA due to the presence of acetaldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH). ALDH and ADH catalyzes the reduction of acetyl-CoA and acetaldehyde to ethanol coupled with the oxidation of NAD(P)H. Multiple alcohol dehydrogenases are present in *T.* BG1. Four *adh* genes, designated *adh*A, *adh*B, *bdh*A, *adh*E, encodes primary alcohol dehydrogenase (P-ADH), secondary alcohol dehydrogenase (S-ADH), butanol dehydrogenase (BDH) and bifunctional alcohol-acetaldehyde dehydrogenase (ADH-ALDH), respectively in *T.* BG1 [Chapter 2]. These alcohol dehydrogenases with overlapping specificities may have different roles and specificity for survival [Radianingtyas and Wright 2003]. The construction of 4 *adh* deficient

mutant strains (*Δadh, ΔadhB, ΔbdhA, Δadh*E) of *T.* BG1 revealed the major function of AdhE isozyme in catalyzing ethanol formation [Chapter 3].

1.3 Regulation of end-product metabolism in thermophilic bacteria

Effect of enzyme regulation: The metabolic flow is determined by a combination of control of synthesis and activity of enzymes in the different pathways. Two classes of regulatory mechanisms may be involved in thermophilic end-product metabolism: those which regulate end-product synthesis in response to the flux of intermediates in the pathway leading to particular end-products, and those which respond to the concentration of the end-products [Lynd 1989]. These mechanisms are not mutually exclusive, and may both have a role. Control of the rate of reactions in enzyme-catalyzed metabolic pathways is different for reversible reactions than for essentially irreversible reactions. The rates of reversible reactions are controlled by substrate and product concentrations. The rates for essentially irreversible reactions are controlled by enzyme activity, and are frequently subject to allosteric control [Lynd 1989]. The majority of the reactions involved with end-product metabolism in thermophiles have been shown to be reversible, especially the reactions involved in the interconversion of the electron carries NAD, NADP, and ferredoxin [Lynd 1989]. Thus the concentration of end-products can be expected to exert an effect on the pattern of end-products formed. According to Slapack *et al.*, the most important factors in regulating end-product ratios in thermophilic anaerobes are alcohol dehydrogenase (ADH), ferredoxin NAD(P) oxidoreductases (FOR), and lactate dehydrogenase (LDH).

Effect of electron flow: In thermophilic bacteria, the pathways of carbon metabolism are inextricably linked with electron transfer reactions. Metabolism of glucose yields NADH from the glycolytic pathway, and a second critical oxidation occurs as pyruvate is converted to acetyl-CoA (Fig. 1.1). The enzyme responsible, pyruvate-ferredoxin oxidoreductase generates reduced ferredoxin (Fd), and the subsequent fate of the electrons is a major determinant of the fermentation pattern. In addition to this enzyme, three other oxidoreductases can transfer electrons between Fd and a donor or acceptor (Fig. 1.2).

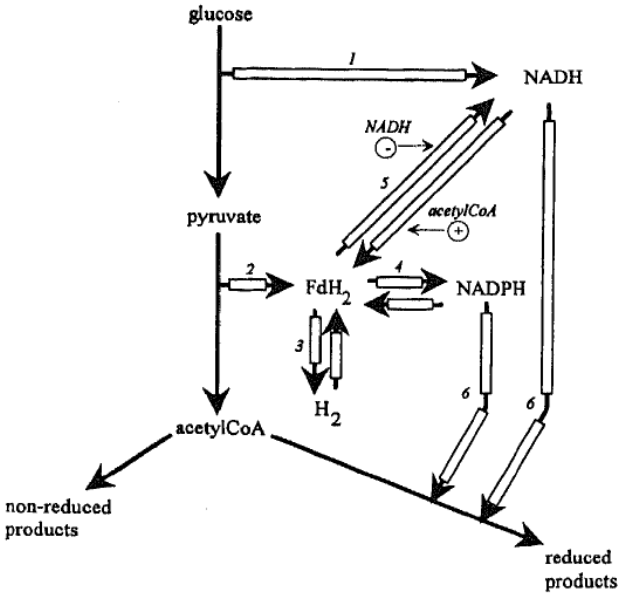

Figure 1.2 Pathway of carbon flow (plain arrows) and electron flow (boxed arrows) in thermophilic bacteria metabolism. 1. Glyceraldehdye 3-P dehydrogenase; 2. Pyruvate-Fd oxidoreductase; 3. Hydrogense; 4. NADPH-Fd oxidoreductase; 5.NADH- Fd oxidoreductase; 6. NAD(P)H-dependent dehydrogenase [Mitchell 1998]

Hydrogenase (Fd-H_2 oxidoreductase) forms hydrogen, and when electrons are lost in this way pyruvate may be metabolized to acetate with the benefit to the cell that additional ATP is generated by the terminal reaction catalysed by acetate kinase (AK). If H_2 cannot be evolved, or H_2 production is limited, electrons must be channelled towards the formation of reduced products. The enzyme NADPH-Fd oxidoreductase may play a major role in generation of NADPH for biosynthesis [Rogers 1986]; however, some NADPH-specific dehydrogenases have been identified [Chen 1993] indicating that it also has a role in formation of fermentation products. The enzyme NADH-Fd oxidoreductase may be used to regenerate NAD^+, but in this direction the reaction involves an unfavourable redox change and requires acetyl-CoA as an activator. The reverse reaction is inhibited by NADH [Jungermann *et al.* 1971, 1973]. The direction of electron flow around reduced ferredoxin may have a critical effect on the nature and quantity of fermentation product.

Consideration of the metabolic scheme indicate that, despite the energetic advantage in acetate formation, it is only possible for all acetyl-CoA to be converted to acetate if glycolytic NADH is reoxidized by H_2 formation via Fd. In practice, H_2 accumulation results in inhibition of hydrogenase activity, thus forcing the cell to produce at least some reduced compounds [Mitchell 1998].

Many thermophilic anaerobic bacteria are able to produce ethanol and lactate, thus providing an alternative means of regenerating NAD^+.

The fermentation profile of any organism is ultimately determined genetically, and the control of enzyme synthesis and enzyme activity is clearly an important factor in regulation of fermentation pathways. Equally important, however, is the control of electron distribution via the enzymes discussed above. The key to understanding the physiology of solvent formation lies in an appreciation of the nature of environmental signals, and how they detected and transmitted to the sites of control which influence cellular metabolism.

1.4 Metabolic engineering of thermophilic bacteria

Metabolic engineering, the deliberate alteration of an organism's metabolism through genetic manipulation [Cameron 1993], often involves amplification or introduction of a naturally occurring metabolic pathway. The full potential of metabolic engineering in the longer term include the creation of new metabolic pathways using enzymes, or modified enzymes, in functions different from those for which they evolved. Metabolic engineering of end-product metabolism has been pursued extensively in *Escherichia coli*, resulting in strains of industrial interest that produce high yields of ethanol [Ingram *et al.* 1998, 1999; Tao *et al.* 2001] as well as other products. Both Gram-positive and thermophilic bacteria have unique advantages compared to Gram-negative bacteria [Dien *et al.* 2003]. However, such metabolic engineering has been pursued only to a limited extent in Gram-positive, mesophilic, obligate anaerobes, and Gram-positive obligate anaerobic thermophiles [Desai *et al.* 2004].

Expression of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in *Clostridium cellulolyticum* resulted in shifts in the distribution of end-products [Guedon *et al.* 2002]. Phosphotransacetylase (*pta*) and butyrate kinase (*bk*) knockout in *Clostridium acetobutylicum* were confirmed genetically, but did not eliminate production of butyrate and acetate [Green *et al.* 1996], perhaps due to the presence of alloenzymes. Antisense RNA complementary of *pta* and *bk* genes has also been used in *C. acetobutylicum* to alter the yields of catabolic end-products [Desai *et al.* 1999]. Moreover, a strain of *Thermoanaerobacterium saccharolyticum* in which the lactate dehydrogenase (*ldh*) gene has been knocked out [Desai et al. 2004] and the further creation of a second strain in which the acetate kinase/phosphotransacetylase (*ack/pta)* gene has also been

knocked out [Shaw *et al.* 2006]. For both strains, gene knockout resulted in undetectable production of the targeted organic acids and resulted in increased ethanol production.

While specifics of how each of these microorganisms was engineered for ethanol fermentation vary, many similarities could be noticed. Following the successful expression of genes needed for ethanol production, ethanol yields could be subsequently improved by eliminating competing reactions. An important step in strain development was integrating the recombinant genes while still maintaining proper gene expression for optimal ethanol production. Furthermore, the eventual success of each engineered microorganism depended upon proper medium optimization and, for some, on long-term adaptation strategies. Finally, careful attention needs to be paid to maintaining a balanced or healthy microbial physiology to ensure hardy cultures and high productivities.

In the study of genetic and metabolic engineering of *T. mathranii* BG1, genetic modification tools has been developed and used for insertion, deletion or over-expression of the target genes. A gene encoding lactate dehydrogenase (LDH) has been deleted, resulting in a significantly improved ethanol producing mutant strain- BG1L1 [Georgieva *et al.* 2007]. A gene encoding pyruvate ferredoxin oxidoreductase (PFOR) has been down-regulated and leads to increased lactate production characteristics and the strain is designated BG1PF1 [Mikkelsen and Ahring 2007]. A gene encoding a hydrogenase or a hydrogenase subunit has been down-regulated and strain BG1H1 was provided with improved hydrogen and acetate production capabilities [Mikkelsen and Ahring 2007]. In addition, deletion of the genes coding for an acetate kinase (AK) and a phosphate acetyltransferase (PTA) eliminate the production of acetate in strain BG1 [Mikkelsen unpublished].

In this PhD study, great efforts have been made on metabolic engineering of strain BG1 for improved ethanol production. The construction of 4 different alcohol dehydrogenase (*adh*) deficient mutant strains (*Δadh, ΔadhB, ΔbdhA, Δadh*E) revealed that the *adh*E deficient mutant (Δ*adh*E) fails to produce ethanol as fermentation product, suggesting the major function of AdhE isozyme in catalyzing ethanol formation in BG1 [Chapter 3]. AdhE was conditionally expressed from a xylose-induced promoter in a recombinant strain (BG1E1) with a concomitant deletion of a lactate dehydrogenase and resulted in an increased ethanol yield with xylose as substrate [Chapter 4].

Furthermore, BG1G1, a derivative strain of BG1, was created by expressing a heterologous NAD^+ dependent glycerol dehydrogenase (GLDH) and a concomitant deletion of *ldh* gene. This mutant showed increased ethanol yield in the presence of glycerol using xylose as a substrate [Chapter 5]. Cofactor manipulation in BG1 was further achieved by both external and genetic strategies to facilitate the regeneration of NADH, which in turn improve the production of reduced product to restore a redox balnce [Chapter 6].

1.5 Alcohol dehydrogenases in thermophilic bacteria

Alcohol dehydrogenases (ADHs) are present in all organisms. They display a wide variety of substrate specificities and play an important role in a broad range of physiological processes. However, it is often difficult to prove their physiological function [Reid *et al.* 1994]. Several classes of ADHs are distinguished based on their cofactor specificity: (i) NAD(P), (ii) pyrrolo-quinoline quinone, heme, or cofactor F420, and (iii) flavin adenine dinucleotide (FAD). The NAD- or NADP-dependent ADHs are often subdivided into three major groups: the zinc-dependent type I ADHs, short-chain type II ADHs, and iron-activated type III ADHs [Reid *et al.* 1994]. Representatives of all three ADH types have been studied extensively at the biochemical and genetic levels in both bacteria and eukaryotes, addressing substrate specificity, metabolic functions and biotechnological potential [Persson *et al.* 1991].

ADHs from thermophiles are of interest because these microbes are uniquely suited for biomass fermentation to ethanol via reduced-pressure distillation, and yield active thermostable enzymes of commercial interest [Radianingtyas and Wright 2003]. Some of the thermophiles are known to contain multiple ADHs, sometimes of different types. The presence of multiple ADHs within one organism conceivably reflects the environment in which the organism has been exposed and adapted. Therefore, each ADH may have a different role and specificity for survival [Radianingtyas and Wright 2003]. Several ADHs have been identified in ethanol producing thermophilic bacteria, including a Zn-dependent ADH in *Thermoanaerobacter brockii* that preferably converts secondary alcohols in the presence of NADP, and several other ADHs in *Thermoanaerobacter ethanolicus* with different proposed metabolic functions in ethanol formation [Radianingtyas and Wright 2003]. For some of these ADHs, a role has been suggested in the disposal of reducting equivalents via the formation of alcohols [van der *et al.* 2001].

Multiple alcohol dehydrogenases (ADHs) are present in *T. mathranii* BG1. Four ADH encoding genes, designated *adh*A, *adh*B, *bdh*A, *adh*E, have been identified in the genome of strain BG1 based on their sequence similarity with the database sequences. The deduced primary structures of these ADHs showed that they are related to iron-activated ADHs (AdhA, BdhA, AdhE) and zinc-containing ADH (AdhB) [Chapter 2]. Almost all the discovered ADHs from thermophilic bacteria and archaea are NAD(P) dependent and classified into three groups, zinc-dependent ADHs, the short-chain ADHs, and the iron activated ADHs [Radianingtyas and Wright 2003]. AdhE comprised two domains of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) and it appears to be the evolutionary product of a gene fusion [Membrillo-Hernandez *et al.* 2000].

AdhA, AdhB, BdhA preferred to use the phosporylated cofactor NADP(H) and AdhE preferred to use NAD(H) as cofactor [Chapter 2]. Coenzyme specificity is an important property for the differentiation of ADHs since NAD(H) is predominantly involved in oxidative reactions for energy conservation whereas NADP(H) is involved in reductive biosynthetic reactions [Chen 1995]. AdhA, BdhA and AdhE showed preference for primary alcohols whereas AdhB converted secondary alcohols faster than the corresponding primary alcohols. Based on their sequence and biochemical properties, AdhA, AdhB, BdhA and AdhE were named primary alcohol dehydrogenase (P-ADH), secondary alcohol dehydrogenase (S-ADH), butanol dehydrogenase (BDH) and bifunctional alcohol/acetaldehyde dehydrogenase (ADH/ALDH), respectively [Chapter 2].

Alcohol dehydrogenases can be involved in a wide range of metabolic processes, the presence in an organism of multiple ADHs with overlapping specificities makes the determination of the specific role of each ADH difficult [Chen 1995]. Kinetic data of AdhA, AdhB, BdhA and AdhE showed higher catalytic efficiency and higher affinity for the substrate and cofactor involved in the reduction reaction than the oxidation reaction, which suggest the function of these ADHs involved in the reduction of aldehyde [Chapter 2]. However, it is difficult from the kinetics to determine which ADH is the most important for ethanol formation in *T. mathranii* BG1. Several factors could influence the metabolic activity by a combination of control of gene synthesis and expressed enzyme activities that involved in the ethanol formation pathway. Thermophilic bacteria employ two different pathways for ethanol production referred to as

Clostridium thermocellum type systems, which contain only NADH-linked primary alcohol dehydrogenase, and *Thermoanaerobacter brockii* type systems, which also contain an NADPH-linked secondary alcohol dehydrogenase activity [Burdette and Zeikus 1994]. Secondary alcohol dehydrogenase in *Thermoanaerobacter ethanolicus* is responsible for most of the ethanol production as primary alcohol dehydrogenase does not contribute significantly to ethanol formation [Bryant *et al.* 1998]. However, these results are obtained without studying other ADHs also present in *Thermoanaerobacter ethanolicus* (BdhA, AdhE). Construction of four *adh* deficient mutant strains of BG1 (*Δadh, ΔadhB, ΔbdhA, Δadh*E) revealed that the *adh*E deficient mutant (Δ*adh*E) fails to produce ethanol as a fermentation product, suggesting the major function of AdhE isozyme in catalyzing ethanol formation in *T. mathranii* BG1 [Chapter 3]. The physiological roles of AdhA, AdhB, and BdhA in *T. mathranii* BG1 remains inconclusive.

Deduced amino acid sequence analysis of *adh*E gene in strain BG1 proposed that its encoded enzyme is an ion containing, NAD(H) dependent bifunctional alcohol/acetaldehyde dehydrogenase (ADH/ALDH) [Chapter 3]. The NH_2- terminal region of this protein is highly homologous to the family of ALDH, whereas the COOH-terminal region is homologous to the family of Fe^{2+}-dependent ADH [Chapter 3]. AdhE appears to be the evolutionary product of a gene fusion [Membrillo-Hernandez *et al.* 2000]. The fusion of ALDH and ADH probably accelerated the successive reduction of acetyl-CoA to ethanol by bringing the two active sites in close proximity. The steady state level of acetaldehyde, a toxic intermediate, could probably be lowered. The sharing of single NAD^+-binding motif on the NH_2-terminal side of the linker could in principle greatly facilitate the sequential catalysis [Membrillo-Hernandez *et al.* 2000]. In summary, the enzyme of AdhE encoded by *adh*E gene in *T. mathranii* BG1 is proposed responsible for the ethanol formation by catalyzing sequential NADH-dependent reductions of acetyl-CoA to acetaldehyde and then to ethanolunder fermentative conditions [Chapter 3].

Moreover, AdhE was conditionally expressed from a xylose-induced promoter in a recombinant strain (AdhE) with a concomitant deletion of a lactate dehydrogenase. Over-expression of AdhE in strain BG1E1 with xylose as a substrate facilitates the production of ethanol at an increased yield. This further confirms the important function of AdhE involved in ethanol production in *T. matharnii* [Chapter 4].

1.6 The role of cofactors for ethanol production

The metabolic pathways leading to the production of most industrially important compounds involve reduction-oxidation (red-ox) reactions. Biosynthetic transformations involving red-ox reactions also offer a considerable potential for the production of fine chemicals over conventional chemical processes. Nicotinamide adenine dinucleotide (NAD) plays a central role in cellular metabolism by functioning as a cofactor in over 300 red-ox reactions [Foster *et al.* 1990] and regulates various enzymes and genetic processes [Berrios-Rivera *et al.* 2002].

The NADH/NAD^+ cofactor pair plays a major role in microbial catabolism, in which a carbon source, such as glucose, is oxidized using NAD^+ as cofactor and producing reducing equivalents in the form of NADH. It is crucially important for continued cell growth that NADH is oxidized to NAD^+ and a balanced redox state is achieved. Under aerobic growth, oxygen is used as the final electron acceptor. While under anaerobic growth, and in the absence of an alternative oxidizing agents, the regeneration of NAD^+ is achieved through fermentation by using NADH to reduce metabolic intermediates and to regenerate NAD^+ (Fig. 1.1). Therefore, in fermentation, alteration in the availability of NADH is expected to have a profound effect on the metabolic network. In addition, studies have shown that the NADH/NAD^+ cofactor pair has a regulatory effect on the expression of some genes and activity of certain enzymes. Examples include, among others, the induction by NADH of the expression of the *adh*E gene that encodes the enzyme alcohol dehydrogenase [Leonardo *et al.* 1993], which catalyzes the production of ethanol during fermentation; and the inhibition of high NADH/NAD^+ ratios on the pyruvate dehydrogenase complex [De Graef *et al.* 1999].

The influence of cofactors in metabolic networks has been evidenced by studies in which the NADH/NAD^+ ratio altered by feeding carbon sources with different oxidation states [Leonardo *et al.* 1996, San *et al.* 2002]; or by expressing an enzyme like NADH oxidase [Lopez de Felipe *et al.*]. The effective regeneration of used cofactors is critical in industrial cofactor dependent production systems due to the high cost of cofactors such as NAD^+. In enzyme bioreactors, NAD^+ dependent formate dehydrogenase (FDH) from yeast and bacteria is extensively used to regenerate NADH from NAD^+ *in vitro*. Cofactor regeneration has been successfully applied *in vitro* for the production of

optically active amino acids [Kragl *et al.*, 1996], esters, alcohols, and other fine chemicals synthesized by different dehydrogenases [Humel and Kula, 1989].

In spite of these advances, biotransformation with the whole cells is still the preferred method for the synthesis of most cofactor-dependents products industrially. In these systems, the cells naturally regenerate the cofactor. However, the enzyme of interest has to compete for the required cofactor with a large number of other enzymes within the cell. Therefore, in cofactor dependent production systems, after the enzyme of interest have been overexpressed, the availability of the required form of the cofactor (reduced or oxidized) can become limiting, making cofactor manipulation crucial for optimal production.

In *T. mathranii* BG1, the pathway of carbon metabolism is inextricably linked with cofactor oxidation and reduction reactions (Fig. 1.1 and 1.2). Catabolism of carbon sources, like glucose and xylose, yields NADH from the glycolytic pathway and converted via pyruvate to the different end-fermentation products. Pyruvate can be converted to either lactate with the oxidation of NADH or to acetyl-CoA and CO_2 with a concomitant reduction of ferredoxin (Fd_{red}). The re-oxidation of Fd_{red} could be catalyzed by different oxidoreductases. NADH and NADPH ferredoxin oxidoreductases catalyse the production of NADH and NADPH, respectively. Hydrogenase (H_2-ferredoxin oxidoreductase) forms hydrogen, and when reducing equivalent was transferred to ferredoxin and released as hydrogen, pyruvate may be metabolized to acetate with the generation of additional ATP. If H_2 can not be evolved, or H_2 production is limited, then NAD^+ could be regenerated to form reduced product – ethanol and lactate. The formation of the final end-fermentation products and their mutual distribution is determined by the response of the cells to a given metabolic state, their need for energy, metabolites for reductive biosynthesis and reducing equivalent.

With a cofactor-dependent ethanol production pathway in *T. mathranii* BG1, it may become crucial to regenerate cofactor to increase the ethanol yield. Overexpression of a heterologous NAD^+ dependent glycerol dehydrogenase (GLDH) and deletion of *ldh* gene in a recombinant BG1 strain (BG1G1) not only eliminate the lactate production, but may also increase the intracellular NADH availability, and this in turn leads to a drastic shift in the metabolic pattern that favored the production of more reduced metabolite, ethanol, under a more reduced environment [Chapter 5]. It is evident that the cell adjusts its partitioning at the acetyl-CoA node by changing the ethanol (consume 2 NADH)

to acetate (consumes no NADH) ratio to achieve a redox balance [Chapter 5]. These findings also support the idea that NADH induces the expression of alcohol dehydrogenase (*adhE*) [Leonardo *et al.* 1993].

Furthermore, the possibility of manipulating the cofactor level in strain BG1 through metabolic engineering provides a way to study metabolites distribution under the different cofactor levels. Cofactor manipulation in BG1 was achieved by both external and genetic strategies [Chapter 6]. The use of a more reduced substrate, mannitol, as a carbon source was shown to increase ethanol production in BG1 wild type and *ldh* deficient strain (BG1L1) than the use of glucose and xylose, due to the increased NADH availability [Chapter 6]. The effect of the reducing power of mannitol can be observed by the dramastic increases in the ethanol-to-acetate ratio (Et/Ac), as a result of imbalanced carbon flux partitioning at the acetyl-CoA node, with more carbon flux directed toward the ethanol pathway [Chapter 6]. Genetic manipulation of the cofactor levels by expressing GLDH that can generate NADH or eliminating an NADH competing pathway or combining both strategies provoked significant changes in metabolite distribution in BG1. With a functional lactate formation pathway, overexpression of GLDH in a recombinant BG1 strain (BG1G2) leads to a significantly decreased ethanol yield accompanied by an increased lactate formation, which was shown to be the preferred route over ethanol production pathway for the regeneration of NAD^+ and the extra reducing equivalents could be transferred to ferredoxin and released as hydrogen [Chapter 6]. However, by inactivating the lactate formation pathway, expressed GLDH in a recombinant BG1 strain (BG1G1) leads to the increased carbon flux channelled towards the production of ethanol to establish redox balance under a more reduced environment. In this case, the additional generated NADH is oxidized via production of ethanol rather than H_2. Therefore, genetic manipulation of *T. mathranii* with improved redox balance should render *T. mathranii* BG1 industrially interesting for ethanol production [Chapter 6].

1.7 Reference

Ben-Bassat A, Zeikus JG. 1981. Saccharide metabolism in *Clostridium thermohydrosulfuricum*: Relation of substrate utilization to enzymatic activities, and comparison of batch and continuous culture fermentations. *Appl Environ Microbiol*

Berrios-Rivera SJ, Bennett GN and San KY. 2002. Metabolic engineering of *Escherichia coli*: increase of NADH availability by overexpressing an $NAD^{(+)}$-dependent formate dehydrogenase. Metab Eng 4, 217-229

Brock TD, Smith DW, Madigan MT. 1984. Biology of microorganisms. Prentice/Hall international Inc. New Jersey

Bryant FO, Wiegel J, and Ljungdahl LG. 1988. Purification and properties of primary and secondary alcohol dehydrogenases from *Thermoanaerobacter ethanolicus*. *Appl Environ Microbiol* 54, 460-465.

Burdette D and Zeikus JG. 1994. Purification of acetaldehyde dehydrogenase and alcohol dehydrogenases from *Thermoanaerobacter ethanolicus* 39E and characterization of the secondary-alcohol dehydrogenase (2 degrees Adh) as a bifunctional alcohol dehydrogenase--acetyl-CoA reductive thioesterase. *Biochem. J* 302 (Pt 1), 163-170.

Burdette DS, Jung SH, Shen GJ, Hollingsworth RI and Zeikus JG. 2002. Physiological function of alcohol dehydrogenases and long-chain (C(30)) fatty acids in alcohol tolerance of *Thermoanaerobacter ethanolicus*. *Appl Environ. Microbiol.* 68, 1914-1918.

Cameron DC. 1993. Cellular and metabolic engineering. *Appl. Biochem. Biotechnol.* 38: 105-140

Chen JS. 1993. Properties of acid and solvenet forming enzymes of clostridia. In: The Clostridia and Biotechnology, pp.51-76. Butterworth-Heinemann, Stoneham, MA.

Chen JS. 1995. Alcohol dehydrogenase: multiplicity and relatedness in the solvent-producing clostridia. *FEMS Microbiol. Rev.* 17, 263-273.

De Graef MR, Alexeeva S, Snoep JL and Teixeira de Mattos MJ. 1999. The steady-state internal redox state (NADH/NAD) reflects the external redox state and is correlated with catabolic adaptation in *Escherichia coli*. *J. Bacteriol.* 181:2351-2357.

Desai RP, Papoutsakis ET. 1999. Antisense RNA strategies for metabolic engineering of *Clostridium acetobutylicum*. *Appl Environ Microbiol*. 65: 936–945

Desai SG, Guerinot ML and Lynd LR. 2004. Cloning of L-lactate dehydrogenase and elimination of lactic acid production via gene knockout in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. *Appl Microbiol. Biotechnol* 65, 600-605.

Dien BS, Cotta MA and Jeffries TW. 2003. Bacteria engineered for fuel ethanol production: current status. *Appl Microbiol. Biotechnol.* 63, 258-266.

Fernando S, Adhikari S, Chandrapal C and Murali N. 2006. Biorefineries: current status, challenges, and future direction, *Energy and Fuels*. Vol. 20, pp.1727–1737

Foster JW, Park YK, Penfound T, Fenger T, and Spector MP. 1990. Regulation of NAD metabolism in *Salmonella typhimurium*: Molecular sequence analysis of the bifunctional nadR regulator and the nadA-pnuC operon. *J. Bacteriol.* 172, 4187–4196.

Georgieva T. 2005. Production of bioethanol from lignocellulosic biomass using thermophilic anaerobic bacteria. Ph.D Thesis. Technical Univeristy of Denmark, Lyngby, Denmark

Georgieva T, Mikkelsen MJ, and Ahring BK. 2007. Ethanol Production from wet-exploded wheat straw hydrolysate by thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1 in a continuous immobilized reactor. *Appl. Biochem. Biotechnol*. 145, 99-110

Green EM, Boynton ZL, Harris LM, Rudolph FB, Papoutsakis ET, Bennett GN. 1996. Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. *Microbiology* 142:2079–2086

Guedon E, Desvaux M, Petitdemange H. 2002. Improvement of cellulolytic properties of *Clostridium cellulolyticum* by metabolic engineering. *Appl Environ Microbiol* 68: 53–58

Hahn-Hagerdal B, Hallborn J, Jeppsson H, Olsson L, Skoog K, and Walfridsson M. 1993. Pentose fermentation to alcohol, p. 231–290. In J. N. Saddler (ed.), Bioconversion of forest and agricultural plant residues. CAB International, Wallingford, United Kingdom

Hinman ND, Wright JD, Hoagland W, and Wyman CE. 1989. Xylose fermentation—an economic analysis. *Appl. Biochem. Biotechnol.* 20/21: 391-401

Hummel H and Kula MR. 1989. Dehydrogenases for the synthesis of chiral compounds. *Eur. J. Biochem.* 184, 1–13.

Ingram LO, Gomez PF, Lai X, Moniruzzaman M, Wood BE, Yomano LP, York SW. 1998. Metabolic engineering of bacteria for ethanol production. *Biotechnol Bioeng* 58: 204–214

Ingram LO, Aldrich HC, Borges ACC, Causey TB, Martinez A, Morales F, Saleh A, Underwood SA, Yomano LP, York SW, Zaldivar J, Zhou SD. 1999. Enteric bacterial catalysts for fuel ethanol production. *Biotechnol Prog* 15:855–866

Jungermann K, Rupprecht C, Ohrloff R, Thauer RK and Dekker K. 1971. Regulation of the reduced nicotinamide adenine dinucleotide-ferredoxin reductase system in *Clostridium Kluyveri*. *J. Biol. Chem.* 246, 960-962

Jungermann K, Thauer RK, Leimenstoll G, Decker K. 1973. Function of reduced pyridine nucleotide ferredoxin oxidoreductases in saccharolytical Clostridia. *Biochem Biophy Act* 305: 268-280

Jones DT, Woods DR, 1991, Clostridia. Plenum Pres, New York and London

Kragl U, Kruse W, Hummel W, and Wandrey C. 1996. Enzyme engineering aspects of biocatalysis: Cofactor regeneration as example. *Biotech. Bioeng.* 52, 309–319.

Lamed R, Zeidus JG. 1980a. Ethanol production by thermophilic bacteria: relationship between fermentation product yields and catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaerobium brockii*. *J Bacteriol* 144: 569-578

Lamed RJ, Zeikus JG. 1980b. Glucose fermentation pathway of *Thermoanaerobacter brokii*. *J Bacteriol* 141: 1251-1257

Leonardo MR, Cunningham PR and Clark DP. 1993. Anaerobic regulation of the adhE gene, encoding the fermentative alcohol dehydrogenase of *Escherichia coli*. *J Bacteriol* 175, 870-878.

Leonardo MR, Dailly Y, and Clark DP. 1996. Role of NAD in regulating the adhE gene of *Escherichia coli*. *J Bacteriol* 178, 6013-6018.

Lopez de Felipe F, Kleerebezem M, de Vos WM and Hugenholtz J. 1998. Cofactor engineering: a novel approach to metabolic engineering in *Lactococcus lactis* by controlled expression of NADH oxidase. *J Bacteriol* 180, 3804-3808.

Lovitt RW, Longin R, and Zeikus JG. 1984. Ethanol production by thermophilic bacteria: physiological comparison of solvent effects on parent alcohol tolerant strains of *Clostridium thermohydrosulphuricum*. *Appl Environ Microbiol* 48 171-177

Lynd LR. 1989. Production of ethanol from lignocellulosic materials using thermophilic bacteria: critical evaluation of potential and review. *Adv Biochem Eng Biotechnol* 38.

Membrillo-Hernandez J, Echave P, Cabiscol E, Tamarit J, Ros J, and Lin ECC. 2000. Evolution of the adhE gene product of *Escherichia coli* from a functional reductase to a dehydrogenase. *J Biol Chem* 275, 33869-33875.

Mitchell WJ. 1998. Physiology of carbohydrate to solvent conversion by clostridia. *Adv Microb Physiol* 39, 31-130.

Mikkelsen MJ, Ahring BK. 2007. World intellectual property organization, WO/2007/134607, *Thermoanaerobacter Mathranii* strain BG1

Persson B, Krook M and Jörnvall H. 1991. Characteristics of short-chain alcohol dehydrogenases and related enzymes. *Eur. J. Biochem.* 200: 537-543.

Radianingtyas H, and Wright PC. 2003. Alcohol dehydrogenases from thermophilic and hyperthermophilic archaea and bacteria. *FEMS Microbiol. Rev* 27, 593-616.

Reid MF and Fewson CA. 1994. Molecular characterization of microbial alcohol dehydrogenases. *Crit Rev Microbiol*, 20, 13–56

Rogers P. 1986. Genetic and biochemistry of *Clostridium* relevant to development of fermentation processes. *Adv Appl Microbiol* 31, 1-60

Slapack GE, Russel I, Stewart GG. 1987. Thermophilic microbes in ethanol production. CRC, Boca Raton FL

Shaw AJ, Desai SG and Lynd LR. 2006. Engineering to improve ethanol production in the xylose utilizing thermophile *Thermoanaerobacterium saccharolyticum* JW/SL-YS485, poster presentation, 28th symposium on biotechnology for fuel and chemicals

Sommer P. 1998. Conversion of hemicellulose and D-xylose into ethanol by the use of thermophilic anaerobic bacteria. Ph.D Thesis. Technical Univeristy of Denmark, Lyngby, Denmark

Sommer P, Georgieva T, Ahring BK. 2004. Potential for using thermophilic anaerobic bacteria for bioethanol production from hemicellulose. *Biochem Soc Trans.* 32(Pt 2), 283-289.

San KY, Bennett GN, Berrios-Rivera SJ, Vadali RV, Yang YT, Horton E, Rudolph FB, Sariyar B and Blackwood K. 2002. Metabolic engineering through cofactor manipulation and its effects on metabolic flux redistribution in *Escherichia coli*. *Metab Eng* 4, 182-192.

Tao H, Gonzalez R, Martinez A, Rodriguez M, Ingram LO, Preston JF, Shanmugam KT. 2001. Engineering a homo-ethanol pathway in *Escherichia coli*: Increased glycolytic flux and levels of expression of glycolytic genes during xylose fermentation. *J Bacteriol* 183:2979–2988

Van der OJ, Voorhorst WG, Kengen SW, Geerling AC, Wittenhorst V, Gueguen Y and de Vos WM. 2001. Genetic and biochemical characterization of a short-chain alcohol dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Eur. J. Biochem* 268, 3062-3068.

Van Zyl C, prior BA, Preez JC. 1989. D-xylose utilization by *Saccharomyces cerevisiae. J Gen Microbiol* 135: 2791-2798

Zeikus JG, Ben-Bassat A, Ng TK, *et al.* 1981. Thermophilic ethanol fermentation. In: Hollaender A (ed) Trends in the biology of fermentations for chemicals and fuels. Plenum Press, New York, pp 441-461

Zubay G. 1988. Glycolysis, glucogeogenesis, and the pentose phosphate pathway. In: Biocehmistry, Macmillan Publishing Company, Collier Macmillan Publishers, New York, London, pp 436-480

Chapter 2

Genetic and biochemical characterization of multiple alcohol dehydrogenases in *Thermoanaerobacter mathranii*

2.1 Abstract

Multiple alcohol dehydrogenases (ADHs) are present in *Thermanaerobacter mathranii* strain BG1. Four genes encoding iron-activated ADHs (AdhA, AdhB, and AdhE) and a zinc-containing ADH (BdhA) are identified in strain BG1. The deduced protein sequences of AdhA, AdhB, BdhA and AdhE correspond to calculated molecular mass of 43 kDa, 38 kDa, 45 kDa and 97 kDa, respectively. All four *adh* genes were functionally expressed in *Escherichia coli* and subsequently purified. AdhA, AdhB, BdhA were characterized as NADP(H) dependent primary ADH, secondary ADH and butanol dehydrogenase (BDH), respectively. Maximal specific activity was observed with 1-propanol for AdhA, 2-propanol for AdhB and butanol for BdhA. AdhE was characterized as an NAD(H) dependent bifunctional alcohol/aldehyde dehydrogenase (ADH/ALDH) that has similar reaction rate on different primary alcohols. All the ADHs in strain BG1, AdhA, AdhB, BdhA and AdhE, have relatively high affinity and reaction rate for acetaldehyde (Km = 0.04, 0.13, 1.60 and 6.18 mM, respectively) in the reduction reaction as opposed to the ethanol oxidation reaction (Km = 58.5, 84.9, 58.1 and 50.8 mM, respectively). The presence of multiple ADHs in *T. mathranii* BG1 with overlapping specificities makes the determination of the specific role of each ADH difficult. The physiological role of multiple ADHs in strain BG1 is also discussed.

2.2 Introduction

Alcohol dehydrogenases (ADH) are present in all organisms. They display a wide variety of substrate specificities and play an important role in a broad range of physiological processes. However, it is often difficult to prove their physiological functions [Reid *et al.* 1994]. Several classes of ADHs are distinguished based on their cofactor specificity: (i)NAD(P), (ii) pyrrolo-quinoline quinone, heme, or cofactor F420, and (iii) flavin adenine dinucleotide (FAD). The NAD- or NADP-dependent ADHs are often subdivided into three major groups: the zinc-dependent type I ADHs, short-chain type II ADHs, and iron-activated type III ADHs [Reid *et al.* 1994]. Representatives of all three ADH types have been studied extensively at the biochemical and genetic levels in both bacteria and eukaryotes, addressing substrate specificity, metabolic functions and biotechnological potential [Persson *et al.* 1991].

ADHs from thermophiles are of interest because these microbes are uniquely suited for biomass fermentation to ethanol via reduced-pressure distillation, and yield active thermostable enzymes of commercial interest [Radianingtyas and Wright 2003]. Some of the thermophiles are known to contain multiple ADHs, sometimes of different types. The presence of multiple ADHs within one organism conceivably reflects the environment in which the organism has been exposed and adapted. Therefore, each ADH may have a different role and specificity for survival [Radianingtyas and Wright 2003]. In several ethanol producing thermophilic bacteria, ADHs have been identified, including a Zn-dependent ADH in *Thermoanaerobacter brockii* that preferably converts secondary alcohols in the presence of NADP, and several other ADHs in *Thermoanaerobacter ethanolicus* with different proposed metabolic functions in ethanol formation [Radianingtyas and Wright 2003]. For some of these ADHs, a role has been suggested in the disposal of reduction equivalents via the formation of alcohols [Van der *et al.* 2001].

Thermoanaerobacter mathranii strain BG1 is a xylanolytic, thermophilic, anaerobic, Gram-positive bacterium originally isolated from an Icelandic hot spring. This organism ferments glucose, xylose, arabinose, galactose and mannose simultaneously and produce ethanol, acetate, lactate, CO_2, and H_2 as fermentation end products [Mikkelsen and Ahring 2007]. The production of ethanol from different carbon substrates by strain BG1 is the result of a series of coordinated enzyme reactions where the final step to ethanol is catalyzed by ADH. This process provides the essential oxidized cofactors for glycolysis,

which is important to maintain the redox balance for the continued cell growth. Strain BG1 appears to produce several ADHs, exploration of the whole genomic DNA sequence revealed that this bacterium contains *adh*A, *adh*B, *bdh*A and *adh*E genes that potentially encode 4 putative ADHs.

The work reported here describes the genetic characterization of these ADHs in the strain BG1 including their sequences analysis and their relatedness to other previously described ADHs. In addition, these 4 ADHs are functionally expressed in *Escherichia coli* and partially purified. Biochemical characterization of these ADHs with respect to substrate specificity, cofactor specificity and kinetics are also described. The possible physiological role of these ADHs in strain BG1 is later discussed.

2.3 Materials and methods

Chemicals and enzymes

All chemicals (analytical grade) were purchased from Sigma-Aldrich (Sigma-Aldrich Danmark A/S, Brøndby, Denmark) or VWR (VWR International ApS, Rødovre, Denmark). The restriction enzymes were obtained from Fermentas (Fermentas GmbH, Germany) and New England Biolabs (Ipswich, England). DNA modification enzymes including T4 polynucleotide kinase (PNK), Calf Intestinal Phosphatase (CIP), T4 DNA ligase were purchased from Fermentas. *Taq* and *pwo* polymerases were purchased from A&A biotechnology (Poland).

Strains, plasmids and DNA manipulation

The strain BG1 was cultured anaerobically at 70^{o}C in anaerobic synthetic BA medium [Larsen *et al.* 1997] supplemented with 2g/L yeast extract and 5g/L xylose or glucose. pUC19 (Invitrogen A/S, Taastrup, Denmark) was used as a cloning vector and plasmid pET30-Ek/Lic or pET-21d (+) (Novagen, USA) were used as expression vectors. *E. coli* Top10 (Invitrogen A/S, Taastrup, Denmark) and Rosetta(DE3)pLysS (Novagen) were used as hosts for cloning and the T7 polymerase-directed overexpression system (pET), respectively. Both strains were grown under standard conditions [Sambrook and Russell 2001]. Plasmid preparation and manipulation, genomic DNA preparation (A&A biotechnology) and transformation were carried out using standard procedures [Sambrook and Russell 2001] or the suppliers' instructions. PCR primers were synthesized and

DNA sequencing was conducted by MWG (MWG-BIOTECH AG, Ebersberg, Germany).

Cloning of the ADH encoding genes

The identification of the genes encoding ADHs was based on automatic annotation of the complete genome sequence of strain BG1 using Bacterial Annotation System (BASys) [Van Domselaar *et al.* 2005]. Four ADHs encoding genes named as *adh*A, *adh*B, *bdh*A and *adh*E were identified in the strain and were PCR amplified from the chromosomal DNA of *T.* BG1 using primers containing *Nde*I/*Pci*I and *Xho*I sites (*italicized*) as listed in Table 2.1. The fragments generated were purified, treated with T4 PNK and ligated with *Sma*I digested and CIP treated pUC19. Following standard subcloning procedure, the obtained plasmids were further digested with NdeI/PciI-XhoI and cloned into the correspondingly digested pET vectors (Table1). Subsequently, the resulting plasmids named pETadhA, pETadhB, pETbdhA, pETadhE were transformed into *E. coli* Rosetta(DE3)pLysS. The sequences of the expression clones were confirmed by sequencing analysis of both DNA strands.

Table 2.1 Primer sequences, amplicon length and expression vectors used in this study

Gene	Primer (5' →3')	Length(kb)	Expression vector
adhA	*CATATG*TGGGAAACTAAAATAAATCCCACCAAG *CTCGAGT*CAGAAAGATTCTTCATAAATCTTGGCAATAAC	1.21	pET-30 Ek/LIC
***adh*B**	C*CATATG*AAAGGTTTTGCAATGCTCAGTATCGG *CTCGAGT*CAAGCTTATGCTAATATTACAACAGGTTTG	1.18	pET-30 Ek/LIC
***bdh*A**	CGGC*ACATGT*GTAAATGCCACATAAAATTAATAATGGAGG CCC*TCGAG*CTACATTGCCATACGTAGTATTTCGGC	1.25	pET-21d (+)
***adh*E**	CGGC*ACATGT*CTACTTTATTACAAGAAAAAAAGGAAAC CCC*TCGAG*TTATTCTCCATAGGCTTTTCTATATATTTC	2.62	pET-21d (+)

Nucleotide sequence analysis

Computer analysis of nucleotide and deduced amino-acid sequences were carried out with Vector *NTI Advance™* Software (Invitrogen). Sequence homology search was conducted using BLAST at www.ncbi.nlm.nih.gov**.**

Production and purification of ADH

Cells of *E. coli* Rosetta(DE3)pLysS harbouring pET-derivatives were grown overnight in 5 ml Luria-Bertani (LB) medium with kanamycin and chloramphenicol (both 50µg/ml) and incubated overnight in a rotary shaker at 37^{o}C. Next, 1 ml of the preculture was used to inoculate 50 ml LB medium with 50µg/ml kanamycin and chloramphenicol in a 100 ml conical flask and

incubated in a rotary shaker at 37°C until an optical cell density at 600nm of 0.6 was reached. The culture was then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and incubation of the culture was continued at 37°C for 5 h in a shaker. Cells were harvested by centrifugation for 20 minutes at 4°C, 4000 rpm, and resuspended in 2 ml extraction buffer (50mM Tris-HCL buffer pH7.0, 10% glycerol and 1mM $MgCl_2$). Cells were disrupted by sonication for 2 min in an ice bath using a digital sonifier (Model 250; Branson Ultrasonics Corporation, Danbury, U.S.A.). The cell extracts were incubated for 15 min at 70°C. Cell debris and denatured *E. coli* proteins were removed by centrifugation at 20000g and 4°C for 30 minutes, the supernatants were used for further analysis.

Enzyme assays

Rates of alcohol oxidation and aldehyde reduction were determined at 70°C, by following either the reduction of NAD^+ or the oxidation of NADH at 340 nm using a Ultrospec 3000 spectrophotometer (Pharmacia Biosystems, Allerød, Denmark), with a temperature-controlled cuvette holder. Each oxidation reaction mixture contained 50 mM Tris-HCL (pH 7.0), 1.2 mM alcohols, and 0.2 mM $NAD(P)^+$. The reduction reaction mixture contained 50 mM Tris-HCL (pH 7.0), 1.2 mM aldehyde or ketone, and 0.2 mM NAD(P)H. In all assays, the reaction was initiated by the addition of an appropriate amount of enzyme. One unit of ADH was defined as the oxidation or reduction of 1 μmol of NAD(P)H or $NAD(P)^+$ per min, respectively. Protein concentration was determined using Bradford reagents (Bio-Rad) with bovine serum albumin as a standard [Bradford 1976].

Kinetics

The AdhA, AdhB, BdhA and AdhE kinetic parameters K_m and V_{max} were calculated from multiple measurements (at least eight measurements) using the Michaelis-Menten equation and Lineweaver-Burk plot [Lineweaver *et al*. 1934]. Other conditions were the same as in the enzyme assay section. All the reactions followed Michaelis-Menten-type kinetics.

2.4 Results

Nucleotide and amino acid sequences analysis

The genome of the strain BG1 has been analyzed for genes encoding putative alcohol dehydrogenases, which resulted in the identification of 4 genes that potentially encode ADHs. Based on their sequences similarity with the known genes, these fours *adh* genes in strain BG1 were named *adh*A, *adh*B, *bdh*A and *adh*E, respectively.

The nucleotide sequence of *adh*A corresponds to an open reading frame (ORF) of 1197 bp, encoding a protein of 399 amino acids with a calculated molecular mass of 43.3 kDa. Blast analysis of *adh*A in strain BG1 showed that it is the most related to the *Thermoanaerobacter ethanolicus adh*A gene (88% nucleotide sequence identity [AF178965]), which encodes a NADP(H) dependent primary alcohol dehydrogenase (P-ADH) that is most active against primary long-chain alcohols [Holt *et al.* 2000]. Blast search analysis [Altschul *et al.* 1997] of its deduced amino-acid sequence revealed that AdhA from strain BG1 is related to a group of iron-containing ADHs. The most similar sequences relative to strain BG1 AdhA include several iron-containing ADHs from *Thermoanaerobactors* with identities of 97% and 98% (Table 2.2). Other similar ADHs from different bacteria and archea with identities from 61% to 75% are listed in table 2.2.

The nucleotide sequence of *adh*B corresponds to an open reading frame of 1056 bp, encoding a protein of 352 amino acids with a calculated molecular mass of 37.8 kDa. The *adh*B gene in strain BG1 is closest to the *Thermoanaerobacter ethanolicus adh*B gene (95% nucleotide sequence identity [U49975]), which encodes a NADP(H) dependent secondary alcohol dehydrogenase (S-ADH) that exhibits significantly greater catalytic efficiency of propan-2-ol oxidation as compared to ethanol oxidation [Burdette *et al.* 1996]. The deduced amino acid sequence of AdhB from strain BG1 has a high degree of sequence identity with a group of zinc-containing ADHs. The most similar sequences relative to strain BG1 AdhB include several zinc-containing ADHs from *Thermoanaerobactors* with identities of 96% and 98% (Table 2.2). Enzymatic activity of *T. ethanolicus* S-ADH requires at least cystine and histine residues and a tightly bound Zn atom [Burdette *et al.* 1996]. Specific proline residues might contribute to *T. ethanolicus* S-ADH thermostability and thermophilicity [Burdette *et al.* 1996]. Other similar ADHs from different bacteria and archea with identities from 68% to 78% are listed in Table 2.2.

Table 2.2 Comparison of *Thermoanaerobacter mathranii* BG1 AdhA, AdhB, BdhA, AdhE with related enzymes

T. BG1 ADHs	Similar enzymes	Length (amino-acid)	Identity	Acc. No./Reference
AdhA (399aa)	*Thermoanaerobacter ethanolicus* Fe-ADH	403	98%	ZP01454905
	Thermoanaerobacter tengcongensis MB4 ADH	342	97%	NP622354
	Caldicellulosiruptor saccharolyticus Fe-ADH	403	75%	YP001179437
	Thermococcus hydrothermalis ADH	406	68%	CAA74334
	Clostridium botulinum Fe-ADH	399	64%	YP001390787
	Clostridium cellulolyticum Fe-ADH	399	61%	ZP01576778.1
AdhB (352aa)	*Thermoanaerobacter ethanolicus* Zn-S-ADH	352	98%	ABC50090
	Thermoanaerobacter brockii ADH	352	97%	P14941
	Thermoanaerobacter tengcongensis Zn-ADH	352	96%	AAM23957
	Clostridium botulinum ($NADP^+$) ADH	353	78%	ABS41960
	Clostridium Beijerinckii ($NADP^+$) ADH	351	76%	AAA23199
	Methanosarcina barkeri str. (NAD^+P) ADH	351	68%	AAZ71266
BdhA (415aa)	*Thermoanaerobacter ethanolicus* Fe-ADH	401	88%	EAO64543
	Carboxydothermus hydrogenoformans (NAD^+) BDH	401	88%	ABB15392
	Geobacter bemidjiensis Fe-ADH	414	66%	EDJ80272
	Chlorobium chlorochromatii (NAD^+) BDH	395	66%	ABB28961
	Prosthecochloris vibrioformis Fe-ADH	395	64%	ABP36969
	Geobacillus thermodenitrificans (NAD^+) BDH	387	44%	ABO68223
AdhE (872aa)	*Thermoanaerobacter ethanolicus* ADH/ALDH	872	96%	EAU56503
	Halothermothrix orenii Fe-ADH/ALDH	887	71%	EAR79503
	Desulfotomaculum reducens Fe-ADH/ALDH	863	65%	ABO50025
	Bacillus weihenstephanensis Fe-ADH/ALDH	867	62%	EAR 75766
	Exiguobacterium sibiricum Fe-ADH	864	60%	EAM88547
	Listeria monocytogenes Fe-ADH	866	59%	EBA23021

ADH, alcohol dehydrogenase; BDH, butanol dehydrogenase; ALDH, aldehyde dehydrogenase; Zn, zinc-containing enzyme; Fe, iron-containing enzyme; $NAD(P)^+$: cofactor dependence of the enzyme; Acc. No., the accession numbers employed by Entrez at the NCBI (when an accession number is not available, known references are given).

The nucleotide sequence of *bdh*A corresponds to an open reading frame of 1245 bp, encoding a protein of 415 amino acids with a calculated molecular mass of 45.0 kDa. The sequence most similar to strain BG1 *bdh*A gene is a gene fragment annotated from the *Carboxydothermus hydrogenoformans* genome [CP000141] with 83% nucleotide identity, that encodes a putative NADH-dependent butanol dehydrogenase A (BdhA). The deduced amino acid sequence of BdhA from *T.* BG1 is related to a group of iron-containing ADHs, mostly NADH-dependent Bdhs. The most similar sequence relative to *T.* BG1 BdhA is a hypothetical iron-containing ADH from *Thermoanaerobacter ethanolicus* with 88% sequence identity (Table 2.2). Less conservation is observed between strain BG1 BdhA and other bacterial ADHs with identities from 44% to 66% (Table 2.2).

The nucleotide sequence of *adh*E corresponds to an open reading frame of 2619 bp, encoding a protein of 872 amino acids with a calculated molecular mass of 96.7 kDa. The alignment of *adh*E sequence to the database using BLAST showed that the *adh*E ORF from strain BG1 has 92% identity to the *adh*E gene of *Thermoanaerobacter ethanolicus* [DQ836061]. The deduced amino acid sequences for *adh*Es among known Gram-positive bacteria are more than 51%

identical based on the sequence alignment (data not shown), and the highest homology was relative to *Thermoanaerobacter ethanolicus* with 96% identity (Table 2.2). AdhE in strain BG1 is related to a group of bifunctional alcohol/acetaldehyde dehydrogenases (ADH/ALDH). The Pfam analysis [Finn *et al.* 2006] indicated that the deduced AdhE of strain BG1 comprised two conserved domains: ADH and ALDH. A single NAD-binding site, which is conserved glycine-rich region with the GXGXXG motif, is located close to the C-terminal region downstream of Aldh and upstream of the N-terminal in Adh [Chapter 3]. The iron binding site with its conserved residues (*HSMAHIL GAKFHLPHGRA)* was found in the ADH domain [Chapter 3].

Cofactor and substrate specificity

AdhA, AdhB, BdhA and AdhE were produced in *E. coli* and partially purified by heating to 70^{o}C, thereby denaturing *E. coli* proteins. The cofactor specificity of AdhA, AdhB, BdhA and AdhE were analyzed using different substrates in the presence of NAD(H) or NADP(H). Table 2.3 showed the specific activity of ADHs using different cofactors in both reduction and oxidation reactions. The results showed that all tested ADHs could use both NAD^+ and $NADP^+$ as cofactors, but with a clear preference. Measurements with AdhA, AdhB and BdhA showed higher specific activity using $NADP^+$ as cofactor, whereas, AdhE showed higher specific activity with NAD^+ as cofactor.

The substrate specificity of AdhA, AdhB, BdhA and AdhE in the oxidation reaction was analyzed using a range of alcohols, including primary alcohols (ethanol, propanol, butanol) and secondary alcohols (2-propanol, 2-butanol), in the presence of preferred cofactors. Table 2.4 presents the substrate specificity of these enzymes in the oxidation reaction. AdhA showed higher activity toward primary alcohols than secondary alcohols. Of the substrates tested, the highest specific activity of AdhA in the oxidative reaction was found with propanol (V_{max}, 4.16 U·mg^{-1}). In contrast, AdhB showed activity towards secondary alcohols that on average were oxidized faster than the primary alcohols. The specific activity of AdhB on secondary alcohols was 4 to 10 fold higher than on the primary alcohols. The highest specific activity of AdhB in the oxidative reaction was found with 2-propanol (V_{max}, 47.22 U·mg^{-1}) as substrate. BdhA was found convert primary alcohols more efficiently than secondary alcohols and showed the highest specific activity on butanol (V_{max}, 3.48 U·mg^{-1}). AdhE showed similar reaction rate on primary alcohols with specific acitivities from

3.64 to 4.01 U·mg^{-1}. Secondary alcohols appeared to be converted less efficiently by AdhE.

Table 2.3. Cofactor specificity of AdhA, AdhB, BdhA, AdhE in both reduction and oxidation reactions

Reaction	Specific activity (U mg^{-1} protein)
AdhA	
Acetaldehyde + NADH + H^+ → Ethanol + NAD^+	0.22 ± 0.03
Acetaldehyde + NADPH + H^+ → Ethanol + $NADP^+$	2.73 ± 0.4
Ethanol + NAD^+ → Acetaldehyde + NADH + H^+	0.47 ± 0.02
Ethanol + $NADP^+$ → Acetaldehyde + NADPH + H^+	2.17 ± 0.2
AdhB	
Acetaldehyde + NADH + H^+ → Ethanol + NAD^+	3.35 ± 0.2
Acetaldehyde + NADPH + H^+ → Ethanol + $NADP^+$	9.18 ± 1.6
Ethanol + NAD^+ → Acetaldehyde + NADH + H^+	1.01 ± 0.05
Ethanol + $NADP^+$ → Acetaldehyde + NADPH + H^+	3.81 ± 0.3
BdhA	
Acetaldehyde + NADH + H^+ → Ethanol + NAD^+	0.94 ± 0.01
Acetaldehyde + NADPH + H^+ → Ethanol + $NADP^+$	7.17 ± 0.8
Ethanol + NAD^+ → Acetaldehyde + NADH + H^+	0.43 ± 0.02
Ethanol + $NADP^+$ → Acetaldehyde + NADPH + H^+	1.17 ± 0.06
AdhE	
Acetaldehyde + NADH + H^+ → Ethanol + NAD^+	8.44 ± 1.1
Acetaldehyde + NADPH + H^+ → Ethanol + $NADP^+$	2.10 ± 0.3
Ethanol + NAD^+ → Acetaldehyde + NADH + H^+	4.46 ± 0.7
Ethanol + $NADP^+$ → Acetaldehyde + NADPH + H^+	1.42 ± 0.1

The compositions of the assay mixtures and condition of the assays are described in "materials and methods".
The average for three independent replicates are shown with their standard deviations.

Table 2.4 Substrate specificity of AdhA, AdhB, BdhA and AdhE in the oxidation reaction

Substrate	**Relative activity (%)**			
	AdhA[a]	**AdhB**[a]	**BdhA**[a]	**AdhE**[b]
Ethanol	100	100	100	100
Propanol	249	104	125	101
Butanol	176	14	245	91
2-propanol	18	1069	38	27
2-butanol	17	476	28	31

Standard assay at 70^{o}C with 100mM listed alcohols as substrate.

[a] The activities of AdhA, AdhB, BdhA were measured with NADP(H)
[b] The activities of AdhE was measured with NAD(H)

Enzyme kinetics

The kinetic properties of AdhA, AdhB, BdhA and AdhE were determined for acetaldehyde reduction and ethanol oxidation. Table 2.5 listed the substrate kinetic parameters for these enzymes using their preferred cofactor. The result showed that all the ADHs in strain BG1 have relatively higher affinity and reaction rate for acetaldehyde in the reduction reaction as opposed to the ethanol in the oxidation reaction. The catalytic efficiency (V_{max}/K_m) of AdhA, AdhB, BdhA and AdhE for acetaldehyde reduction was also much higher than that for ethanol oxidation.

Table 2.5. Substrate kinetic parameters of AdhA, AdhB, BdhA and AdhE

Enzyme	Substrate	K_m (mM)	V_{max} (nmol/min)	V_{max}/K_m (nmol/min/mM)
AdhA[a]	Ethanol	57.5	6.2	0.11
	Acetaldehyde	0.04	19.9	497.5
AdhB[a]	Ethanol	84.9	13.3	0.16
	Acetaldehyde	0.13	76.3	586.9
BdhA[a]	Ethanol	58.1	1.9	0.033
	Acetaldehyde	1.6	31.4	19.6
AdhE[b]	Ethanol	50.8	3.8	0.075
	Acetaldehyde	6.2	13.8	2.23

[a] The activities of AdhA, AdhB, BdhA were measured with NADP(H)
[b] The activities of AdhE was measured with NAD(H)

2.5 Discussion

In *T. mathranii* BG1, four ADH genes designated *adh*A, *adh*B, *bdh*A, *adh*E, have been identified in the genome based on their sequence similarity with the database sequences. The deduced primary structures of these ADHs showed that they were related to iron-activated ADHs (AdhA, BdhA, AdhE) and zinc-containing ADH (AdhB). Almost all the discovered ADHs from thermophilic bacteria and archaea are NAD(P) dependent and classified into three groups, zinc-dependent ADHs, the short-chain ADHs, and the iron activated ADHs [Radianingtyas and Wright 2003]. AdhE comprised two domains of ADH and ALDH and it appears to be the evolutionary product of a gene fusion [Membrillo-Hernandez *et al.* 2000].

AdhA, AdhB, BdhA preferred the phosporylated cofactor NADP(H) and AdhE preferred NAD(H) as cofactor. Coenzyme specificity is an important property for the differentiation of ADHs since NAD(H) is predominantly involved in oxidative reactions for energy conservation whereas NADP(H) is involved in reductive biosynthetic reactions [Chen 1995]. AdhA, BdhA and AdhE showed preference for primary alcohols whereas AdhB was converted secondary alcohols faster than the corresponding primary alcohols. Based on

their sequence and biochemical properties, AdhA, AdhB, BdhA and AdhE were named primary alcohol dehydrogenase (P-ADH), secondary alcohol dehydrogenase (S-ADH), butanol dehydrogenase (BDH) and alcohol/aldehyde dehydrogenase (ALDH/ADH) in *T. mathranii* BG1.

ADHs can be involved in a wide range of metabolic processes, the presence in an organism of multiple ADHs with overlapping specificities makes the determination of the specific role of each ADH difficult [Chen 1995]. Kinetic data showed higher catalytic efficiency for the reduction reaction and higher affinity for the substrate and cofactor involved in this reaction, which suggest that AdhA, AdhB, BdhA and AdhE are all involved in the reduction of aldehydes. However, it is difficult from the kinetics to determine the role of each ADH in ethanol formation in *T. mathranii* BG1. Several factors could influence the metabolic activity by a combination of control of gene synthesis and expression of enzyme activities that are involved in the ethanol formation pathway. Thermophilic bacteria employ two different pathways for ethanol production referred to as type I *Clostridium thermocellum* type system, which contain only NADH-linked primary ADH, and *Thermoanaerobacter brockii* type systems, which also contain NADPH-linked secondary ADH activity [Burdette and Zeikus 1994]. The secondary ADH in *Thermoanaerobacter ethanolicus* is responsible for most of the ethanol production as primary ADH does not contribute significantly to ethanol formation [Bryant et al. 1988]. However, these results are obtained without studying other ADHs also present in *Thermoanaerobacter ethanolicus* (BdhA, AdhE in Table 2.2). Chapter 3 shows that an *adh*E deficient mutant BG1 strain (Δ*adh*E) fails to produce ethanol as fermentation product, suggesting the major function of AdhE isozyme in catalyzing ethanol formation in the strain. More experiments are required to establish the physiological roles of AdhA, AdhB, and BdhA in *T. mathranii* BG1.

2.6 References

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254

Bryant FO, Wiegel J and Ljungdahl LG. 1988. Purification and properties of primary and secondary alcohol dehydrogenases from *Thermoanaerobacter ethanolicus*. *Appl Environ Microbiol* 54: 460-465

Burdette D and Zeikus JG. 1994. Purification of acetaldehyde dehydrogenase and alcohol dehydrogenases from *Thermoanaerobacter ethanolicus* 39E and characterization of the secondary-alcohol dehydrogenase (2 degrees Adh) as a bifunctional alcohol dehydrogenase--acetyl-CoA reductive thioesterase. *Biochem J 302: 163-170*

Burdette DS, Vieille C and Zeikus JG. 1996. Cloning and expression of the gene encoding the Thermoanaerobacter ethanolicus 39E secondary-alcohol dehydrogenase and biochemical characterization of the enzyme. *Biochem J* 316:115-122.

Chen JS. 1995. Alcohol dehydrogenase: multiplicity and relatedness in the solvent-producing clostridia. *FEMS Microbiol Rev* 17, 263-273

Finn RD, Mistry J, Schuster-Böckler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M, Khanna A, Durbin R, Eddy SR, Sonnhammer ELL and Bateman A. 2006. *Nucleic Acids Research* Pfam: clans, web tools and services, Database Issue 34:D247-D251

Holt PJ, Williams RE, Jordan KN, Lowe CR and Bruce NC. 2000. Cloning, sequencing and expression in *Escherichia coli* of the primary alcohol dehydrogenase gene from *Thermoanaerobacter ethanolicus* JW200. *FEMS Microbiol. Lett.* 190: 57-62

Larsen L, Nielsen P and Ahring BK. 1997. *Thermoanaerobacter mathranii* sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland. *Arch. Microbiol.* 168: 114-119

Lineweaver H, and Burk D. 1934. The determination of enzyme dissociation constants. *J Am Chem Soc* 56: 658—666

Membrillo-Hernandez J, Echave P, Cabiscol E, Tamarit J, Ros J and Lin ECC. 2000. Evolution of the adhE gene product of *Escherichia coli* from a functional reductase to a dehydrogenase. *J Biol Chem* 275: 33869-33875

Mikkelsen MJ, Ahring BK. 2007. World intellectual property organization, WO/2007/134607, *Thermoanaerobacter Mathranii* strain BG1

Persson B, Krook M, and Jörnvall H. 1991. Characteristics of short-chain alcohol dehydrogenases and related enzymes. *Eur. J. Biochem.* 200:537-543.

Radianingtyas H and Wright PC. 2003. Alcohol dehydrogenases from thermophilic and hyperthermophilic archaea and bacteria. *FEMS Microbiol. Rev.* 27: 593-616.

Reid MF and Fewson CA. 1994. Molecular characterization of microbial alcohol dehydrogenases. *Crit. Rev. Microbiol.*, 20: 13–56

Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Stephen FA, Madden TL, Schäffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res.* 25:3389-3402

Van Domselaar GH, Stothard P, Shrivastava S, Cruz JA, Guo A, Dong X, Lu P, Szafron D, Greiner R, Wishart DS. 2005. BASys: a web server for autpmated bacterial genome annotation. *Nucleic Acids Res. J*. 33(Web Server issue):W455-9

Van der OJ, Voorhorst WG, Kengen SW, Geerling AC, Wittenhorst V, Gueguen Y. and de Vos WM. 2001. Genetic and biochemical characterization of a short-chain alcohol dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Eur. J. Biochem* 268: 3062-3068

Chapter 3

Effect of deleting alcohol dehydrogenase encoding gene on ethanol production in *Thermoanaerobacter mathranii*

3.1 Abstract *Thermoanaerobacter mathranii* BG1 contains four genes namely *adh*, *adh*B, *bdh*A and *adh*E encoding 4 different alcohol dehydrogenases with overlapping specificities. Sequence analysis showed high similarity to previously described primary alcohol dehydrogenase (P-ADH), secondary alcohol dehydrogenase (2-ADH), butanol dehydrogenase (BDH) and bifunctional aldehyde-alcohol dehydrogenase (ALDH-ADH). To investigate the roles of these ADHs in ethanol production in strain BG1, 4 *adh* deficient mutant strains (*Δadh, ΔadhB, ΔbdhA, Δadh*E) were constructed and the corresponding phenotype was characterized. The observation that the *adh*E deficient mutant (Δ*adh*E) fails to produce ethanol as fermentation product reveals the major function of the AdhE isozyme in catalyzing ethanol formation in *T. mathranii* BG1. The inability of Δ*adh*E to produce ethanol resulted in increased acetate and lactate production. Δ*adh*E was defective in aldehyde dehydrogenase acitivity, but still remains alcohol dehyrogenase activity. The deduced amino acid sequence of *adh*E comprised conserved ALDH and Fe^{2+}-ADH domains with a single NAD^{+} binding site. AdhE is proposed to be an ion containing, NAD/NADH dependent bifunctional alcohol/acetaldehyde dehydrogenase that is responsible for ethanol production in *T. mathranii* BG1.

3.2 Introduction

Thermoanaerobacter mathranii strain BG1 is a xylanolytic, thermophilic, anaerobic, Gram-positive bacterium originally isolated from an Icelandic hot spring. This organism ferments glucose, xylose, arabinose, galactose and mannose simultaneously and produce ethanol, acetate, lactic acid, CO_2, and H_2 as fermentation end products [Mikkelsen and Ahring 2007]. Strain BG1 is of interest because of its broad carbohydrate utilization range, high temperature growth optimum and its potential use for production of ethanol from biomass. This organism could grow and produce ethanol from hemicellulose hydrolysate of wheat straw with the same ethanol yield as found for synthetic medium [Georgieva *et al.* 2007].

Metabolic engineering of end-product metabolism has been pursued extensively in *Escherichia coli*, resulting in strains of industrial interest that produce high yields of ethanol as well as other products [Desai *et al.* 2004]. Both Gram-positive and thermophilic bacteria have unique advantages compared to Gram-negative bacteria [Dien *et al.* 2003]. However, such metabolic engineering has been pursued only to a limited extent in Gram-positive, mesophilic, obligate anaerobes, and Gram-positive obligate anaerobic thermophiles [Desai *et al.* 2004]. Recently, tools for genetic modification of the thermophilic anaerobe have been developed and used for insertion, deletion or over-expression of the target genes in strain BG1. The gene encoding lactate dehydrogenase (*ldh*) had been deleted, resulting in a significantly improved ethanol producing mutant strain - BG1L1 [Georgieva *et al.* 2007]. Furthermore, great efforts have been made on genetic modification of strain BG1 to optimize its ethanol production. These genetically modified organisms have played a crucial role in our understanding of both genetic and metabolic functions of *T. mathranii* BG1.

Among the end products of fermentation in *T. mathranii* BG1, ethanol arises from acetyl-CoA due to the presence of acetaldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH). ALDH and ADH catalyze the reduction of acetyl-CoA and acetaldehyde to ethanol coupled with the oxidation of NADH and provides NAD^+ for glycolysis, which is essential for the continued cell growth. Strain BG1 appears to produce several ADHs with overlapping specificities. Exploration of the whole genomic DNA sequence of the organism revealed that this bacterium contains *adh*, *adh*B, *bdh*A and *adh*E genes that

encodes 4 different ADHs [Chapter 2]. The alignment of these sequences in database using BLAST [Basic Local Alignment Search Tool] revealed that they are close to previously described primary alcohol dehydrogenase (P-ADH), secondary alcohol dehydrogenase (S-ADH), butanol dehydrogenase (BDH) and bifunctional aldehyde-alcohol dehydrogenase (ALDH-ADH), respectively [Chapter 2].

Although the sequences of 4 different *adh* genes have been analyzed and their corresponding product, ADHs, been characterized [Chapter 2], but it still remains unclear about the detailed function of each ADH in the ethanol formation in strain BG1. In addition, several factors could also influence on metabolic activity by combination of control of gene synthesis and expressed enzyme activity in the ethanol formation pathway. In the work reported here, we sought to further investigate these ADHs and their roles in ethanol production by metabolic engineering. This was done by knocking out each individual *adh*, *adh*B, *bdh*A and *adh*E gene from the genome of strain BG1 to create 4 *adh* deficient mutants, *Δadh, ΔadhB, ΔbdhA, Δadh*E. The mutant altered in ethanol production pathway was characterized and the enzyme responsible for ethanol production was identified.

3.3 Materials and methods

Culture origin, maintenance and cultivation

T. mathranii BG1 was originally isolated from an Icelandic hot spring. The strain was cultured anaerobically at 70^{o}C in anaerobic synthetic BA medium as previously described [Larsen *et al.* 1997]. The medium was further supplemented with 2g/L yeast extract and 5g/L xylose or glucose was added as the growth substrate. Single colony was isolated using the anaerobic roll-tube technique [Hungate 1969, Bryant 1972] with medium solidified with Phytagel and $MgCl_2$. Colonies were picked with a sterile needle, cultured and stored in 25% glycerol and 75% growth medium at -80^{o}C in 2-ml sealed vials under 80%N_2/20%CO_2 atmosphere. Culture recovered from glycerol stocks were grown in liquid medium prior to use in experiments. For selection of antibiotic-resistant strains, the medium was supplemented with 50 μg/ml kanamycin and the culture was maintained anaerobically at 70^{o}C.

Plasmids and PCR primers

Cloning vector pUC19 was obtained from invitrogen (Invitrogen A/S, Taastrup, Denmark) and PCR primers were synthesized by MWG (MWG-BIOTECH AG, Ebersberg, Germany). The primers were designed based on the upstream and downstream sequences of the deleted *adh* gene, internal sequences of P2 promoter and *htk* marker gene.

Primers used in this work were:

adh 5F: 5'CCCACCAAGATTTTTGAACTTCGGTGC3'

adh 5R: 5'GCGGCCGCAGCGCTCTCGAGGATAGAGGCACTCATAAGCTATTGCAGG3'

adh 3F: 5'CTCGAGAGCGCTGCGGCCGCCCCAATCTCTTAATCCAAAGGCTATTCTG3'

adh 3R: 5'AGCTTCTAAATCCTCGCCACCTCC3'

*adh*B 5F: 5'GCTTAACAATACATACTGCAAATTGCCTATG 3'

*adh*B 5R: 5'GCGGCCGCAGCGCTCTCGAGGGTACAACAACGCGATCACCAGG3'

*adh*B 3F: 5'CTCGAGAGCGCTGCGGCCGCGGGGACTTAGTCCCCCATTTTTATGC3'

*adh*B 3R: 5'GAATAGGCGGAGGCCTTGGTATTG3'

*bdh*A 5F: 5'CAACAGCCGACCAGAGCACATCA3'

*bdh*A 5R: 5'GCGGCCGCAGCGCTCTCGAGCTGTCCCACAAACCTGCATCCTC3'

*bdh*A 3F: 5'CTCGAGAGCGCTGCGGCCGCGGAGGAGGTAGATAGTGATGAGCAAC3'

*bdh*A 3R: 5'TAGCTCTCTTCACAGCCCTGCC3'

*adh*E 5F: 5'GCTTATGCCTACTTTATTACAAGAAAAAAAGGAAAC3'

*adh*E 5R: 5'GCGGCCGCAGCGCTCTCGAGCAATGTAGCAGGGGACATTGCCAG3'

*adh*E 3F: 5'CTCGAGAGCGCTGCGGCCGCGGGTAAATTTTCAGTTTGCCCTTATTCTATTTCC3'

*adh*E 3R: 5'GAGGATGGGTTAAAGCAGAGGACATTG3'

p2F: 5'GCAGCAATTGACTAGAAGCTTTGACACC3'

htkR: 5'GAGCACTGCAGCGTAACCAACATG3'

adh exR:5'CTGGGGGCATTCGATGACACACC3'

*adh*B exR:5'GGCGGGCAAGCTGTTGGTTTCC3'

*bdh*A exF: 5'CCTCTTCCCGACAGAAAGGAGATG3'

*bdh*A exR:5'CCTCCATAGCCCGCCAAGCAC3'

*adh*E exR: 5'GTTGAGGATGTAAATTCTGCTATGTACGG3'

DNA preparation, manipulation

DNA fragments were subcloned using standard protocols [Sambrook and Russell 2001]. *E. coli* Top10 (Invitrogen A/S, Taastrup, Denmark) transformants were grown in Luria-Bertani medium supplemented with ampicillin or kanamycin. Plasmid preparation and manipulation, genomic DNA preparation (A&A biotechnology, Poland) and transformation were carried out using standard procedures or the suppliers' instructions. Restriction enzymes

were obtained from Fermentas (Fermentas GmbH, Germany) and New England Biolabs (Ipswich, England). *Taq* and *pfu* polymerase were purchased from Fermentas and DNA sequencing were conducted in MWG.

Knockout vector contruction

The knockout vector (Fig. 3.1) was designed based on pUC19. The DNA region flanking the 5' and 3'-ends of the target *adh* genes were amplified from genomic DNA of strain BG1 using primers listed above. The name of the listed primers composed of the gene name (*adh*, *adh*B, *bdh*A and *adh*E), DNA flanking region (5'-end or 3'-end) and the direction of the primers (F: forward R: reverse). Of the four primers used in amplifying the DNA flanking region of the target gene, two primers were designed to have sequence tags (5'CTCGAGAGCGCTGCGGCCGC3') complementary to each other. Fusion PCR was performed using the first step PCR products as template with terminal primers (Fig. 3.2A). PCR amplification was performed with *Taq* and *pfu* polymerases. The resulting fusion PCR fragments were treated with T4 PNK (T4 polynucleotide kinase from Fermentas) and cloned into *Sma*I digested, CIP (Calf Intestinal Phosphatase from Fermentas) treated pUC19 plasmid. Following the standard subcloning procedure, the obtained construct was further digested with *Eco47*III, treated with CIP and ligated to kanamycin marker cassette (*htk*) which is amplified by primers p2F-htkR and treated with T4 PNK. Following standard subcloning prodcedure, construct PadhHTK was obtained. Restriction analysis and DNA sequencing confirmed the presence and the orientation of the cloned *adh-up, adh-down* fragments as well as *htk* marker gene.

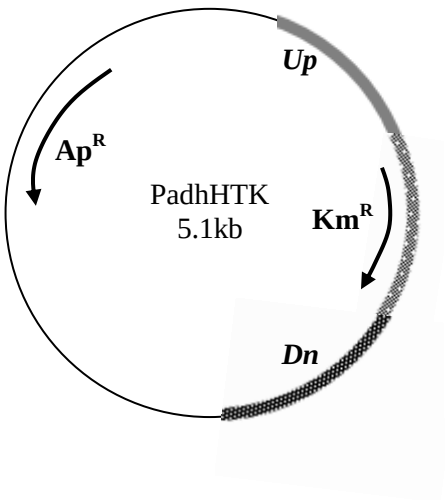

Figure 3.1 Construction of pUC19 based *T.* BG1 *adh* knockout vector Padh:HTK. Km, *htk* gene conferring kanamycin resistance; Ap, *bla* gene conferring ampicillin resistance; *up* and *dn*, upstream and downstream sequence of *adh* gene.

Multiple displacement amplification (MDA) and enzyme digestion

PadhHTK plasmid DNA (10ng-100ng) was placed into 0.2 ml tubes in a total volume of 48 µl containing 33 mM Tris-acetate (pH7.9), 10 mM Mg-acetate, 66 mM K-acetate, 0.1% (v/v) Tween 20, 1 mM DTT, 0.4 mM dNTPs and 50 µM exonuclease-resistant hexamer (Fermentas). The mixture was heat treated at 95°C for 2 min and chilled to 4°C. A final concentration of 1unit/ml of yeast pyrophosphatase (Fermentas) and 300u/ml phi29 DNA polymerase (Fermentas) was added into the mixture described above to bring the final volume to 50 µl. The reactions were incubated at 30°C for 5 h and terminated by heating to 65°C for 10 min. 3 µg of amplified DNA products from MDA reaction using PadhHTK as templates was later digested with *Sca*I.

Transformation of T. mathranii BG1

Digested DNA products after the MDA reaction were transformed into strain BG1 using electroporation as described previously [Tyurin *et al.* 2004]. Electropulsed cell suspensions were recovered and cultured in liquid BA medium with 5g/L glucose and later transferred into the same medium supplemented with 50µg/ml kanamycin. The cultures grown with kanamycin were later transferred into roll tubes for single colony isolation.

Determination of ADH and ALDH activity

The tested strains were cultivated in 100 ml of BA media with 5g/L xylose as growth substrate at 70°C under anaerobic conditions. Cultures at an OD578 of ~0.5 were harvested by centrifugation of 50 ml of the culture at 40000 rpm and 4°C for 30 min. The pellet was suspended in 2 ml of ice chilled extraction buffer composed of 50 mM Tris-HCL, 10% glycerol and 1 mM $MgCl_2$ at pH 8.0. The cells were sonicated for 2 min in an ice bath (Digital Sonifier: Model 250; Branson Ultrasonics Corporation, Danbury, U.S.A.). The sonicated cells were centrifuged at 20000 g and 4°C for 30 min. The supernatant was used for enzyme activity assay. The ADH and ALDH activity was determined as previously described [Soboh *et al.* 2004]. ADH and ALDH activity was assayed in both the forward and the reverse direction by measuring the formation of NAD(P)H at 340 nm at 70°C. One unit of activity was defined as the amount of enzyme that forms 1 µmol NAD(P)H per minute. Total concentration in the cell

extracts was routinely measured by the method of Bradford [Bradford 1976] using bovine serum albumin (BSA) as a standard.

Fermentation

All fermentation experiments were performed as batch fermentations under strictly anaerobic conditions using 10% (v/v) inoculum. 26 ml serum tubes were used in the experiment with 10 ml of BA media supplemented with 5g/L glucose or xylose. The triplicate cultures were inoculated with overnight culture and a sample after inoculation was saved for analysis. The cultures were grown at 70^{o}C and the samples were collected after 48 h of growth. To measure the growth rate of the tested strain, cell density at OD 578nm was measured every 60 min during the exponential growth phase.

Analytical techniques

Cell density (OD) was measured at 578nm in a spectrophotometer (Milton Roy Spectronic 301, Bie & Berntsen A-S, Denmark). Samples for sugars and fermentation products determination were prepared and analyzed using HPLC as described previously [Georgieva *et al.* 2007].

3.4 Results

Chromosomal integration and isolation of Δadh, ΔadhB, ΔbdhA, ΔadhE strains

The *adh*-null strains were constructed by homologous recombination (Fig 3.2 B.C). The DNA fragments used for homologous recombination were created by 2-step PCR. The *htk* marker cassette was connected with the 500-bp genome regions at the 5' or 3'end of the *adh* ORF. The fragments were transformed into strain BG1 and the transformants were recovered with growth observed after 2 days in cultures transformed with *adh*, *adh*B, *bdh*A and *adh*E deletion constructs. Five of each presumptive chromosomal integrant isolates were picked as single colonies from the roll tubes. All picked isolates grew in liquid medium containing kanamaycin and yielded identical results following the PCR analysis (data not shown). One of each isolates was chosen for further study and the obtained *adh* deficient strain is described below as Δ*adh*, Δ*adh*B, Δ*bdh*A, Δ*adh*E.

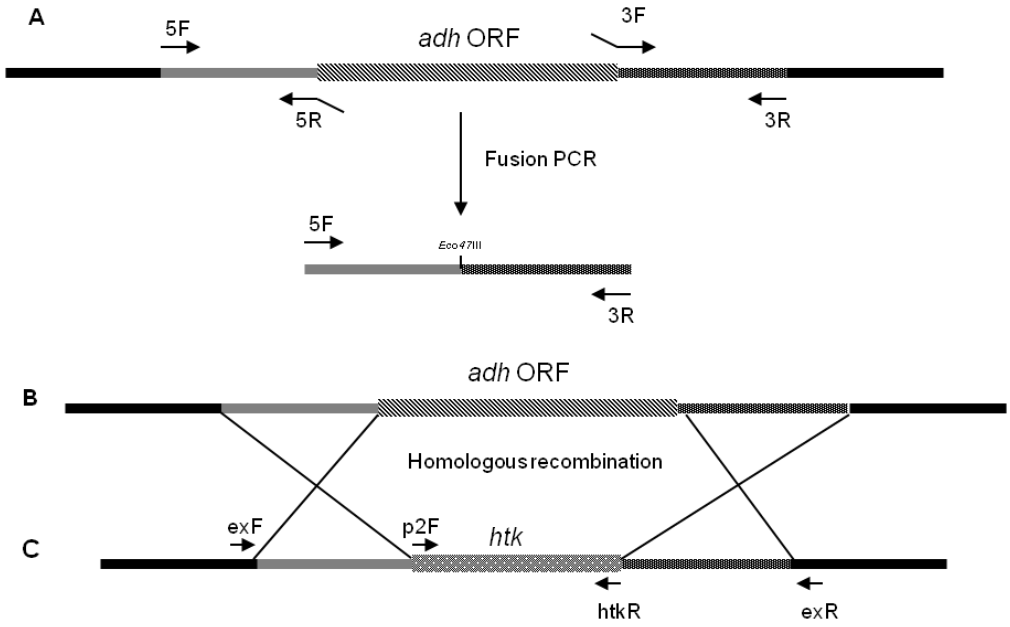

Figure 3.2 A. The two-step PCR method used for connecting 5' and 3' end of *adh* ORF; **B.** Strain BG1 *adh* and flanked upstream, downstream region. **C.** Map of double crossover region with *Δadh* and inserted *htk* gene.

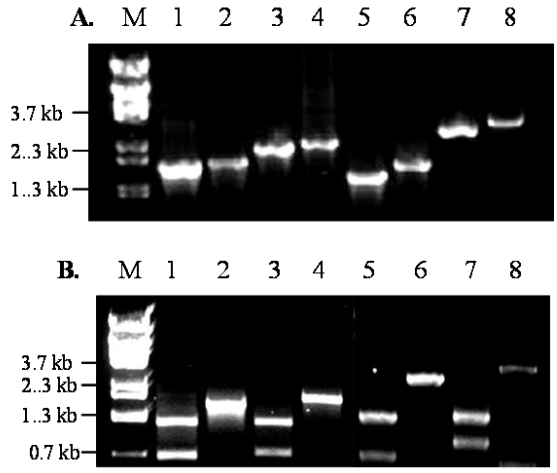

Figure 3.3 A. PCR analysis of *Δadh, ΔadhB, ΔbdhA, Δadh*E mutant strains (lane 1-4) compared with wild type (lane 5-8) using exF and exR primers shown in Figure 3.2. **B.** *Bgl*II digestion of the PCR products on *Δadh, ΔadhB, ΔbdhA, Δadh*E mutant strains (lane 1.3.5.7) compared with wild type (lane 2.4.6.8)

Molecular characterizaiton of Δadh, ΔadhB, ΔbdhA, ΔadhE strains

Gene disruption in Δ*adh*, Δ*adh*B, Δ*bdh*A, Δ*adh*E mutant strains were verified by PCR using genomic DNA as template compared with wild type. The primers exF and exR of the corresponding *adh* ORF (Fig. 3.2C) were used in PCR reactions and yielded 1.94, 2.01, 2.40, 2.46kb fragments (Fig. 3.3A, lane 1-4), which are the expected size for chromosomal integration of corresponding *adh*, *adh*B, *bdh*A and *adh*E genes. In contrast, controls with wild-type genomic DNA using the same PCR mix were also conducted and resulted in the fragments with expected sizes of 1.66, 1.87, 2.80, 3.44kb (Fig. 3.3A, lane 5-8). In addition, all the yielded PCR products from both wild type and the mutants were further digested with *Bgl*II, an enzyme restriction site that is only present in the *htk* gene. As shown in Fig. 3.3B, the digested pattern of all the mutant strains resulted in 2 bands (Fig. 3.3B, lane 1.3.5.7) with expected sizes in contrast to only 1 band (Fig. 3.3B, lane 2.4.6.8) in the wild type. Furthermore, all the PCR products were also sequenced and confirmed the replacement of the *adh* ORF with the *htk* marker cassette via a double-crossover event (Fig. 3.2 B.C) in Δ*adh*, Δ*adh*B, Δ*bdh*A, Δ*adh*E.

Fermentation study of adh deficient strains

Fermentation study were performed with wild type and *adh* deficient mutants, *Δadh, ΔadhB, ΔbdhA, ΔadhE*, to study the effect of eliminating each ADH on the metabolic pattern of strain BG1. BG1 wild type strain and *Δadh, ΔadhB, ΔbdhA, ΔadhE* mutant strains were grown anaerobically in batch for 48 h on glucose and xylose. Fig. 3.4 shows the results of these experiments, including the concentration of consumed substrates (mM) and the concentration of different metabolites produced (mM) at the end of fermentation. A comparison of the results for the wild type and *adh* deficient strains showed that *Δadh, ΔadhB* had similar metabolic pattern as seen in wild type, but an increase of 15% ethanol yield was seen in *ΔbdhA* strain on both substrates. However, *ΔadhE* showed the most prominent changes in metabolic products distribution, with no ethanol production and high levels of lactate and acetate on both substrates. From these results, it is concluded that *adhE* was essential for the ethanol production in strain BG1 and thus the *adh*E deficient strain, *ΔadhE*, was further studied as described in the following experiments.

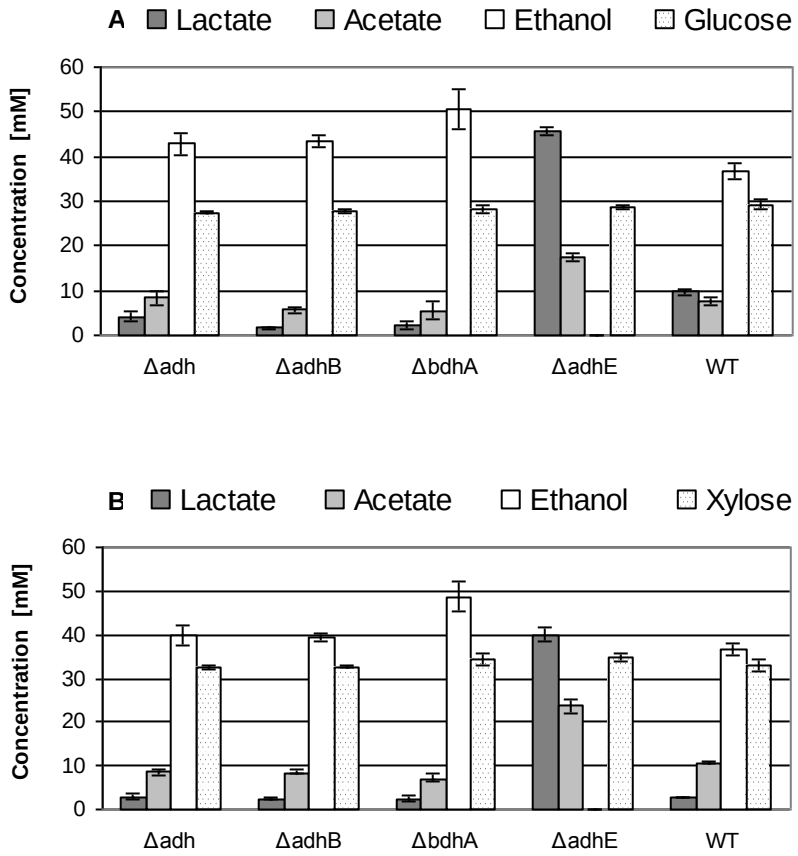

Figure 3.4 Results of fermentation experiment for BG1 wild type strain and *adh* deletion mutant strains showing the concentration of consumed substrates and produced metabolites. **A** glucose; **B** xylose. Results are from anaerobic experiment using 5g/L of the carbon source in BA medium after 48 h of culture. Values shown are of triplicate cultures.

Table 3.1 End-product analysis of glucose and xylose fermentation by wild-type and *ΔadhE*

	Glucose		Xylose	
Strain	Wild type	*ΔadhE*	Wild type	*ΔadhE*
Substrate (mM)	29.3 ± 1.07	28.7 ± 0.30	32.8 ± 1.40	34.9 ± 0.95
Lactate (mM)	9.7 ± 0.62	45.5 ± 0.96	2.9 ± 0.06	40.1 ± 1.64
Acetate (mM)	7.7 ± 0.82	17.3 ± 0.90	10.6 ± 0.59	23.8 ± 1.23
Ethanol (mM)	36.8 ± 1.68	ND	36.4 ± 1.38	ND
$Y_{L/S}$	0.33	1.59	0.09	1.55
$Y_{A/S}$	0.26	0.60	0.32	0.68
$Y_{E/S}$	1.25	-	1.11	-
μ_{max}	0.238 ± 0.018	0.057 ± 0.002	0.076 ± 0.003	0.034 ± 0.001
C recovery (%)	92 ± 0.85	109 ± 2.23	91 ± 2.56	110 ± 2.15

mutant strains.

Results are from anaerobic experiment using 5g/L of the carbon source in BA medium after 48 h of culture. Values shown are of triplicate cultures. ND-Not detected.

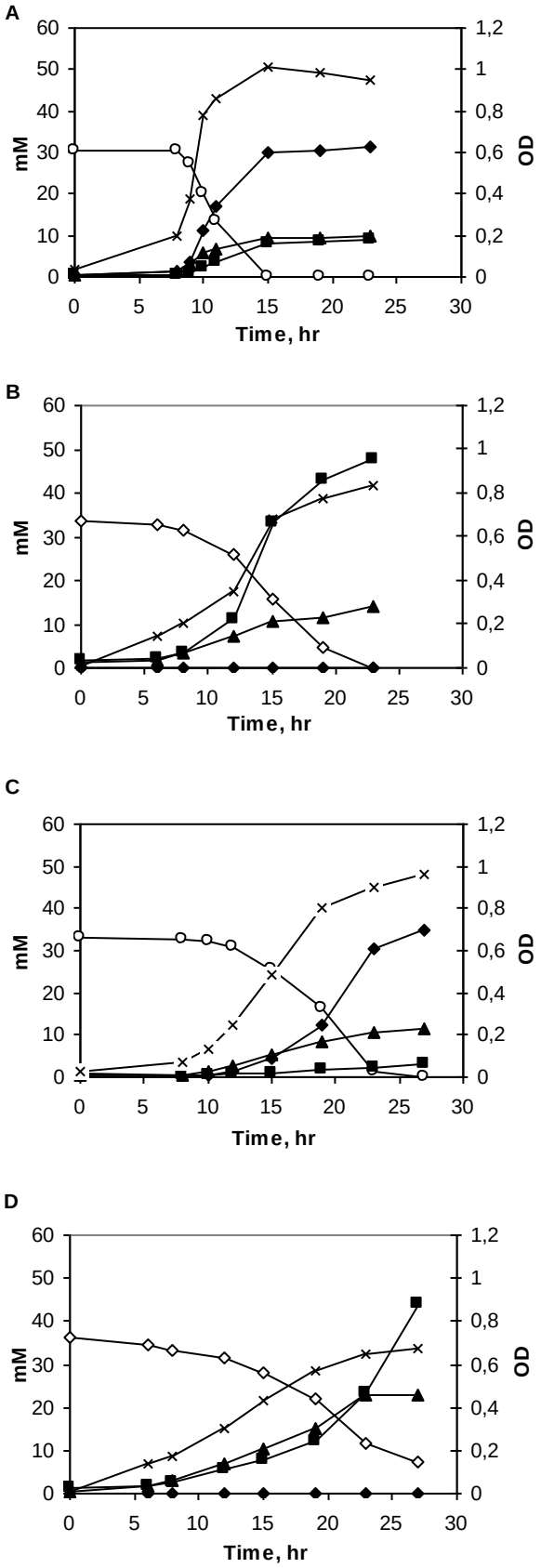

Figure 3.5 A-D Growth curves for BG1 wild type strain and *ΔadhE* mutant strain on glucose and xylose. **A.** Wild type, glucose; **B.** *ΔadhE*, glucose; **C.** wild-type, xylose; **D.** *ΔadhE*, xylose. Substrate (o), lactate (■), acetate (▲), ethanol (♦) and OD (x)

Physiological characterization of strain ΔadhE

Substrates consumption, fermentation product formation, product yields, maximum specific growth rate and carbon recovery are tabulated in Table 3.1 for batch cultures of BG1 wild type strain and *ΔadhE* mutant strain. Fig 3.5 presents representative batch growth curves for BG1 wild type strain and *ΔadhE* mutant strain on glucose (Fig. 3.5: A. B) and xylose (Fig. 3.5: C. D). Two other replicate experiments showed similar trends (data not shown). Compared with wild type, the maximum specific growth rate of strain *ΔadhE* was reduced and the final cell density was also decreased. When *ΔadhE* was grown on glucose or xylose in the absence of selective pressure, ethanol was below the limit of detection at all times tested, but was readily detected for growth of the wild type. In addition, *ΔadhE* showed a higher level of organic acid production than the wild type, with acetate yield increased of 130% and 113% on glucose and xylose, respectively. Produced lactate in *ΔadhE* was fivefold and seventeen fold higher on glucose and xylose, respectively, compared to the wild type.

Enzyme study of strain ΔadhE

To investigate the function of AdhE in the ethanol formation in strain BG1, the activities of the two key enzymes involved in ethanol formation, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) were determined using cell extracts from wild type and strain *ΔadhE*. Table 3.2 lists these results with specific activities of ADH and ALDH, involved in the interconversion of acetyl-CoA, acetaldehyde and ethanol using different cofactors. The experiments with ADH activity tests showed that cell extracts of the wild type catalyzed the NADH-dependent acetaldehyde reduction and NADP-dependent ethanol oxidation. Cell extracts from *ΔadhE* showed similar ADH activities and specific rates. Determination of ALDH activity in wild type cell extracts showed both NADH and NADPH-dependent acetyl-CoA reduction, but no activity was detected in the reverse reaction. In contrast, *ΔadhE* cell extracts did not show any of the ALDH activity in the same context.

Table 3.2 Rates of reactions specific for alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) from the cell extracts in BG1 wild type strain and *ΔadhE* mutant strain. ND: Not detected.

Enzyme and reaction	**Activity (units/mg)**	
Adh	**Wild type**	*ΔadhE*
Acetaldehyde + NADH → ethanol + NAD^+	0.19 ± 0.03	0.14 ± 0.02
Acetaldehyde + NADPH → ethanol + $NADP^+$	ND	ND
Ethanol + NAD^+ → acetaldehyde + NADH	ND	ND
Ethanol + $NADP^+$ → acetaldehyde + NADPH	0.10 ± 0.02	0.13 ± 0.03
Aldh		
Acetyl-CoA + NADH → acetaldehyde + NAD^+	0.21 ± 0.04	ND
Acetyl-CoA + NADPH → acetaldehyde + $NADP^+$	0.24 ± 0.02	ND
Acetaldehyde + CoA + NAD^+ → acetyl-CoA + NADH	ND	ND
Acetaldehyde+ CoA + $NADP^+$ → acetyl-CoA + NADPH	ND	ND

Note. One unit is defined as the amount of enzyme that produced 1µmol of NADH per minute at 70 ^oC and pH 7.0. Values shown are average of triplicates from anaerobic cultures. ND: not detected (less than 0.001U/mg)

*adh*E *gene in T. mathranii* BG1

*adh*E gene in *T. mathranii* BG1 is 2619 bp long and encodes a 872 amino acid polypeptide with an estimated mass of 96.7 kDa. The alignment of *adh*E sequence to the database using BLAST showed that *adh*E ORF from strain BG1 has 92% identity relative to the *adh*E gene of *Thermoanaerobacter ethanolicus* ATCC 31550. The deduced amino acid sequence for *adh*E among known Gram-positive bacterial are more than 51% identical based on the sequence alignment (data not shown). The most similar sequence is that of *Thermoanaerobacter ethanolicus* ATCC 33223 with 98% similarity and 95% identity. The Pfam analysis [Finn *et al.* 2006] indicated that the deduced AdhE of strain BG1 comprised two conserved domains, namely an alcohol dehydrogense (ADH) domain and an aldehyde dehydrogenase (ALDH) domain (Fig 3.6). NAD^+-binding sites, which are conserved glycine-rich region with a GXGXXG motif, are not in every bacterium analyzed [Membrillo-Hernandez *et al.* 2000]. AdhE in strain BG1 appears to contain a single NAD^+ binding site located close to the C-terminal region downstream of ALDH and upstream of the N-terminal in ADH (Fig. 3.6). The iron binding site with its conserved residues is also seen in the ADH domain (Fig. 3.6).

```
  1    MPTLLQEKKE TKEKTEVKET TDVKKQIDLL VERAKRAQEK FMSYTQEQID

 51    EIVKVMALAG IEKHVELAKL AHEETKMGVY EDKITKNLFA VEYVYNYIKD

101    KKTVGILSEN LEENHMEVAE PVGIIAGVTP VTNPTSTTMF KCLIAIKTRN

151    PIIFSFHPKA LKCSIEAAKT MYEAALKAGA PEGCIGWIET PSIEATQLLM

201    THPGVSLILA TGGAGMVKAA YSSGKPALGV GPGNVPCYIE KTANIKRAVS

251    DLILSKTFDN GTVCASEQAV IIDEEIANEV KKLMREYGCY FLNKEETKKL

301    EEFAIDKNTG LMNPAVVGQP AVKIAQMAGF KVPENTKILV AEYPAVGPKY

351    PLSREKLSPI LALYTVKDYK EGIKRCEEMI QFGGLGHSAV IHSENQQIIN

                                          NAD+ binding site
401    EFANRVQASR ILVNAPASQG AIGDIYNTAT PSLTLGCGTM GGNSTTDNVS

          Linker
451    VYNLINIKRV FIRKERMKWF RVPPQIYFER GSIQYLSQVK GKKAFIVTDP

501    AMVKLGFVDK VTYQLDKANI KYEIFSEVEP DPSVDTVEKG IKIMKEFEPD

551    LLIAVGGGSA IDAAKGMWLF YEYPDTKFED LRLKFMDIRK RTYRFPELGK

601    KALFIAIPTT SGTGSEVTAF AVITDKKKNI KYPLTDYELT PDIAIIDPDF

651    VMTIPPSVTA DTGMDALTHA IEAYVSVMAS DYTDALAEKA IKLIFEYLPK

                                          Fe2+ binding site
701    AYKNGQDKVA REKMHNASCI AGMAFTNAFL GINHSMAHIL GAKFHLPHGR

751    ANAILLPYVI EYNAELPKKF ASFPQYEYPK AAEKYAEIAK FLGLPASTTD

801    EGVKSLIEAI KNLMKELNLP LTLKDAGINK EEFEKQIMEM ADIAFNDQCT

851    GSNPRMPLVS EIAEIYRKAY GE
```

Figure 3.6 AdhE amino acid sequence and putative binding sites. The acetaldehyde dehydrogenase (ALDH) and iron containing alcohol dehydrogenase (ADH) domains are connected by a proposed linker (underlined). The NAD-binding site is located on the basis of the GXGXXG motif. The iron binding site is shown with its conserved residues italicized.

3.5 Discussion

To study the metabolic contribution of *adh*, *adh*B, *bdh*A and *adh*E to the alcohol fermentation in *T. mathranii* BG1, the corresponding *adh* null mutant strains, Δ*adh*, Δ*adh*B, Δ*bdh*A, Δ*adh*E, were constructed and characterized. Data from PCR and sequencing results confirmed that a homologous recombination-mediated knockout of each *adh* gene had occurred via a double-crossover event in each mutant.

Fermentation studies of the *adh* deficient strains showed that Δ*adh*, Δ*adh*B strains grew fermentatively like the wild-type cells with similar metabolic pattern and growth rate, suggesting that *adh*, *adh*B genes are not essential in the ethanol formation and fermentative growth of strain BG1. An increase in the ethanol yield by Δ*bdh*A is possibly due to the loss of the ethanol oxidation activity of BdhA. A previous study has shown that *adh*, *adh*B, *bdh*A genes encode NADP(H) dependent enzymes and have greater catalytic efficiency towards primary, secondary alcohols and butanol, respectively [Chapter 2]. However, further studies will be necessary to elucidate the detailed function of *adh*, *adh*B, *bdh*A in strain BG1. The major changes in ethanol formation were seen in strain Δ*adh*E. The inability of Δ*adh*E to produce ethanol underscores the important role of *adh*E in ethanol production. *adh*E was found to encode a bifunctional aldehyde-alchol dehydrogenase (ALDH-ADH) catalyzing the interconversion of acetyl-CoA, acetaldehyde and ethanol with preference of NAD(H) as cofactor [Chapter 2]. Δ*adh*E strain was further studied as discussed in the following sections.

The Δ*adh*E mutant strain, deficient in *adh*E and ethanol formation, implies that the AdhE protein is the major isozyme in catalyzing ethanol production in strain BG1. Elimination of ethanol as fermentation product resulted in proportionately increased yields of acetate and lactate. This is because the absence of *adh*E eliminated effective NADH oxidation by ALDH and ADH activities which provide essential NAD^+ substrate for the key glycolytic enzymes and associated ATP production [Clark 1989]. Thus more lactate were produced to maintain the redox balance with alternative NADH oxidation by lactate dehydrogenase (LDH) and more acetate was produced possibly due to the increased level of acetyl-CoA. Δ*adh*E featuring with retarded cell growth, reduced growth rate and decreased cell density support the essential role of AdhE for the anaerobic growth of strain BG1. In addition, strain Δ*adh*E was

defective in acetaldehyde dehydrogenase acitivity, but still maintained the alcohol dehydrogenase activity, suggesting that AdhE is an important enzyme for ethanol formation and appears to be the major ALDH in the cell. The remaining ADH activities are possibly catalyzed by other ADHs present in the cell and acetyl-CoA reduction rather than acetaldehyde reduction seems to be the rate-limiting step in Δ*adh*E. In *C. acetobutylicum* ATCC824, which is primarily used on large-scale butanol production, AdhE is an important enzyme for butanol formation and inactivation of the *adh*E gene resulted in a mutant strain showed drastic reduction of solvent formation [Green and Bennett, 1996]. These results are comparable since AdhE enzyme in strain BG1 exhibits a high degree of similarity to AdhE in *C. acetobutylicum* ATCC824 encoded by *aad* gene [Nair *et al.* 1994] (data not shown).

The deduced amino acid sequence of the *adh*E gene in strain BG1 suggests that its encoded enzyme is an ion containing, NAD(H) dependent bifunctional alcohol/acetaldehyde dehydrogenase. The NH_2-terminal region of this protein is highly homologous to a family of ALDH, whereas the COOH-terminal region is homologous to the family of Fe^{2+}-dependent ADHs. AdhE appears to be the evolutionary product of a gene fusion [Membrillo-Hernandez *et al.* 2000]. The fusion of ALDH and ADH probably accelerated the successive reduction of acetyl-CoA to ethanol by bringing the two active sites in close proximity. The steady state level of acetaldehyde, a toxic intermediate, could probably be lowered. The sharing of single NAD-binding motif on the NH_2-terminal side of the linker (Fig. 3.6) could in principle greatly facilitate the sequential catalysis [Membrillo-Hernandez *et al.* 2000]. In summary, the AdhE protein encoded by *adh*E gene in *T. mathranii* BG1 is here proposed to be responsible for the ethanol formation by catalyzing sequential NADH-dependent reductions of acetyl-CoA to acetaldehyde and then to ethanol under fermentative conditions.

3.6 References

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biocehm* 72: 248-254.

Bryant MP. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. *Am J Clin Nutr.* 12:1324–1328.

Clark DP. 1989. The fermentation pathways of *Escherichia coli*. *FEMS Microbiol. Rev.* 5: 223-234

Desai SG, Guerinot ML and Lynd LR. 2004. Cloning of L-lactate dehydrogenase and elimination of lactic acid production via gene knockout in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. *Appl Microbiol. Biotechnol* 65, 600-605.

Dien BS, Cotta MA and Jeffries TW. 2003. Bacteria engineered for fuel ethanol production: current status. *Appl Microbiol. Biotechnol.* 63, 258-266.

Finn RD, Mistry J, Schuster-Böckler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M, Khanna A, Durbin R, Eddy SR, Sonnhammer ELL and Bateman A. 2006. Pfam: clans, web tools and services. *Nucleic Acids Research.* Database Issue 34:D247-D251

Georgieva T, Mikkelsen MJ and Ahring BK. 2007. Ethanol production from wet-exploded wheat straw hydrolysate by thermophili anaerobic bacterium *Thermoanaerobacter* BG1L1 in a continuous immobilized reactor. *Appl Biochem Biotech* 145, 99-110

Green EM and Bennett GN. 1996. Inactivation of an aldehyde/alcohol dehydrogenase gene from *Clostridium acetobutylicum* ATCC 824. *Appl Biochem Biotechnol* 57-58, 213-221.

Hungate RE. 1969. A roll tube method for the cultivation of strict anaerobes. *Methods Microbiol* 3B, 117-132.

Larsen L, Nielsen P and Ahring BK. 1997. *Thermoanaerobacter mathranii* sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland. *Arch. Microbiol.* 168, 114-119.

Membrillo-Hernandez J, Echave P, Cabiscol E, Tamarit J, Ros J and Lin ECC. 2000 Evolution of the adhE gene product of *Escherichia coli* from a functional reductase to a dehydrogenase. *J Biol Chem* 275, 33869-33875.

Mikkelsen MJ, Ahring BK. 2007. World intellectual property organization, WO/2007/134607, *Thermoanaerobacter Mathranii* strain BG1

Nair RV, Bennett GN and Papoutsakis ET. 1994 Molecular characterization of an aldehyde/alcohol dehydrogenase gene from *Clostridium acetobutylicum* ATCC 824. *The J Bacteriol* 176, 871-885.

Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Soboh B, Linder D and Hedderich R. 2004. A multisubunit membrane-bound [NiFe] hydrogenase and an NADH-dependent Fe-only hydrogenase in the

fermenting bacterium *Thermoanaerobacter tengcongensis*. *Microbiology* 150, 2451-2463.

Tyurin MV, Desai SG and Lynd LR. 2004. Electrotransformation of *Clostridium thermocellum*. *Appl Environ. Microbiol* 70, 883-890.

Chapter 4

Effect of overexpressing a bifunctional aldehyde/alcohol dehydrogenase on ethanol production in *Thermoanaerobacter mathranii*

4.1 Abstract A native bifunctional alcohol/acetaldehyde dehydrogenase (AdhE) has been identified as an enzyme responsible for ethanol production in *Thermoanaerobacter mathranii*. In this study, AdhE was conditionally expressed from a strong regulated promoter in *T. mathranii* strain BG1. A recombinant BG1 strain, BG1E1, with the regulated expression of *adh*E gene was constructed by knocking out its native lactate dehydrogenase gene (*ldh*). The expression level of *adh*E gene in strain BG1E1 was controlled by the P_{xyl} promoter to increase the transcriptional level by the presence of xylose, which is confirmed by RT-PCR. The deletion of *ldh* gene in BG1E1 eliminated the lactate production. The up-regulation of *adh*E transcription in BG1E1 resulted in increased ethanol production despite the effect of lactate elimination. Overexpression of AdhE in strain BG1E1 with xylose as a substrate facilitates the production of ethanol at an increased ethanol yield. Therefore, identification of the gene responsible for ethanol production provides a partial elucidation of the ethanol metabolism pathway in *T. mathranii* and facilitates the construction of an improved strain with a high ethanol yield by genetic engineering.

4.2 Introduction

Thermoanaerobacter mathranii strain BG1 is a xylanolytic, thermophilic, anaerobic, Gram-positive bacterium, was originally isolated from an Icelandic hot spring. This organism ferments glucose, xylose, arabinose, galactose and mannose simultaneously and produce ethanol, acetate, lactic acid, CO_2, and H_2 as fermentation end products [Mikkelsen and Ahring 2007]. Strain BG1 is of interest because of its broad carbohydrate utilization range, high temperature growth optimum and its potential use for produciton of ethanol from biomass. This organism could grow and produce ethanol from hemicellulose hydrolysate of wheat straw with the same ethanol yield as found for synthetic medium [Georgieva *et al.* 2007].

Among the end products of fermentation in strain BG1 (Fig. 4.1), ethanol arises from acetyl-CoA due to the presence of acetaldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH). ALDH and ADH catalyzes the reduction of acetyl-CoA and acetaldehyde to ethanol coupled with the oxidation of NADH and provides NAD^+ essentials for glycolysis, which is important to maintain the redox balance for the continued cell growth. *T. mathranii* BG1 appears to produce several ADHs with overlapping specificities. Exploration of the whole genomic DNA sequence of the organism revealed that this bacterium contains *adh*, *adh*B, *bdh*A and *adh*E genes that encode a primary alcohol dehydrogenase (P-ADH), a secondary alcohol dehydrogenase (S-ADH), a butanol dehydrogenase (BDH) and a bifunctional aldehyde-alcohol dehydrogenase (ALDH-ADH), respectively (Chapter 2).

Metabolic engineering of end-product metabolism has been pursued extensively in *Escherichia coli*, resulting in strains of industrial interest that produce high yields of ethanol as well as other products [Desai SG *et al.* 2004]. Recently, genetic modification tools had been developed and used for insertion, deletion or overexpression of the target genes in strain BG1. Furthermore, great efforts have been made on metabolic engineering of *T. mathranii* BG1 for improved ethanol production. The gene coding for lactate dehydrogenase (*ldh*) has been deleted, resulting in a significantly improved ethanol producing mutant strain- BG1L1 (*Δldh*) [Georgieva *et al.* 2007]. The construction of 4 *adh* deficient mutant strains (*Δadh, ΔadhB, ΔbdhA, Δadh*E) revealed that the *adh*E deficient mutant strain (Δ*adh*E) fails to produce ethanol as fermentation

product, suggesting the major function of the AdhE isozyme is to catalyze ethanol formation in *T. mathranii* BG1 [Chapter 3].

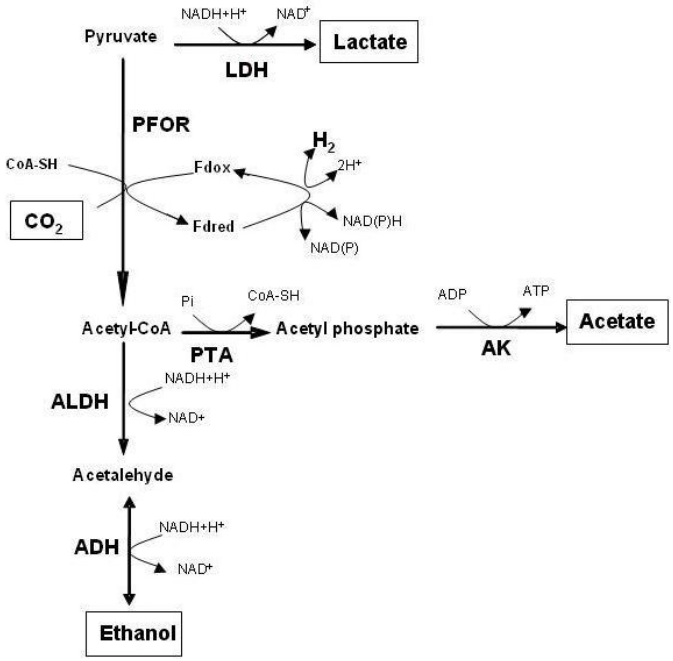

Figure 4.1 Products formation pathway in thermophilic anaerobic ethanol producing bacteria. LDH= Lactate dehydrogenase, PFOR= Pyruvate-ferredoxin oxidoreductase, PTA:=Phosphotransacetylase, AK= Acetate kinase, ALDH= Acetaldehyde dehydrogenase, PDC=Pyruvate decarboxylase, ADH= Alcohol dehydrogenase

The *adh*E gene in strain BG1 is 2619 bp long and encodes a 872 amino acid polypeptide with an estimated mass of 96.7 kDa [Chapter 2]. The deduced primary structure of AdhE comprises two domains of ADH and ALDH and it appears to be the evolutionary product of a gene fusion [Membrillo-Hernandez *et al.* 2000]. AdhE contain a single NAD^+ binding site and an iron binding site [Chapter 3]. AdhE is NAD(H) dependent and related to iron-activated ADH [Chapter 2]. Functional production of AdhE in *E. coli* showed both bidirectional ALDH and ADH activities. Kinetic studies showed that AdhE has higher affinity and reaction rate for acetaldehyde in the reduction reaction as opposed to the ethanol oxidation [Chapter 2]. AdhE is an important enzyme for ethanol formation and appears to be the major ALDH in *T. mathranii* BG1 [Chapter 3].

This paper investigates a genetic means of regulating the expression of *adh*E gene in strain BG1 and studies its effect on ethanol formation by metabolic engineering. In this study, *adh*E gene was integrated into the chromosome of strain BG1 by knocking out the native *ldh* gene. The aim was to control the

expression of the inserted *adh*E gene using the P_{xyl} promoter, which is activated when xylose is present in the medium. Genetic manipulations are made by cloning of the *adh*E gene into the constructed vector (Fig 4.2) and subsequently transformed into strain BG1 to target the *ldh* gene of the host cell.

4.3 Materials and methods

Culture origin, maintenance and cultivation

Strain BG1 was originally isolated from an Icelandic hot spring. The strain was cultured anaerobically at 70°C in anaerobic synthetic BA medium as previously described [Larsen *et al.* 1997]. The medium was further supplemented with 2g/L yeast extract and 5g/L xylose or glucose as the growth substrate. Single colony was isolated using the anaerobic roll-tube technique [Hungate 1969, Bryant 1972] with medium solidified with Phytagel and $MgCl_2$. Colonies were picked with sterile needle, cultured and stored in 25% glycerol and 75% growth medium at -80°C in 2-ml sealed vials under 80%N_2/20%CO_2 atmosphere. Culture recovered from glycerol stocks were grown in liquid medium prior to use in experiments. For selection of antibiotic-resistant strains, the medium was supplemented with 50µg/ml kanamycin and the culture was maintained anaerobically at 70°C.

Plasmids and DNA manipulation

Plasmid p3KpT was used as the sources of *ldh* deletion vector that conferring thermostable kanamycin resistance. Plasmid pGapadhEK was used as the standard in the real time PCR analysis. Plasmid preparation and manipulation, genomic DNA preparation (A&A biotechnology) and transformation were carried out using standard procedures [Sambrook and Russell 2001] or the suppliers' instructions. PCR primers were synthesized and DNA sequencing was conducted by MWG (MWG-BIOTECH AG, Ebersberg, Germany).

Cloning of adhE gene

The constructed vector, 8.60kb p3KpadhET (Fig. 4.2), was based on plasmid p3KpT. *adh*E gene with ribosome binding site (2641bp) was PCR amplified from genomic DNA of strain BG1 using primer adhEF1 (5'AAGGAGGAC-TGCATATGCCTACTTTATTACAAGAAAAAAAGG3') and adhER1 (5'GAAAT-ATATAGAAAAGCCTATGGAGAATAACTCGAGGG3'). PCR amplification

was performed with *Taq* and *pwo* polymerase. The resulting PCR fragments were treated with T4 polynucleotide kinase (Fermentas GmbH, Germany) and cloned into *Sma*I digested p3KpT plasmid after treated with calf intestinal phosphatase (Fermentas). Following standard subcloning procedure, constructed p3KpadhET was obtained. Restriction analysis and DNA sequencing confirmed the presence and orientation of the cloned *adh*E gene, *htk* kanamycin marker as well as the flanking regions.

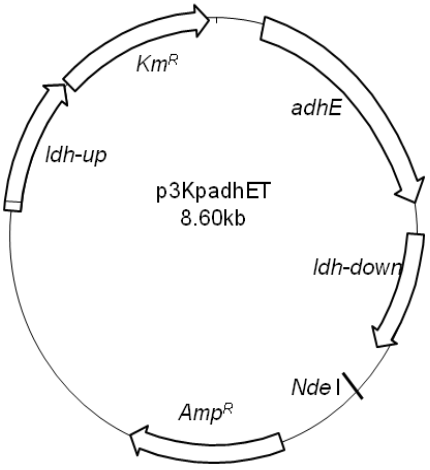

Figure 4.2 Construction of xylose controlled regulative expression vector p3KpadhET. K_m, kanamycin marker gene; *Amp*, ampicillin marker gene; *ldh-up* and *ldh-down*, *ldh* upstream and downstream DNA fragments; *adh*E, bifunctional alcohol /acetaldehyde dehydrogenase; rbs, ribosome binding site; P_{xyl}, xylose promoter.

Multiple displacement amplification (MDA) and enzyme digestion

Plasmid DNA (10ng-100ng) was placed into 0.2 ml PCR tubes in a total volume of 48 µl containing 33 mM Tris-acetate (pH7.9), 10 mM Mg-acetate, 66 mM K-acetate, 0.1% (v/v) Tween 20, 1 mM DTT, 0.4 mM dNTPs and 50 µM exonuclease-resistant hexamer (Fermentas). The mixture was heating treated at 95°C for 2 min and chilled to 4°C in PCR System Thermocycler. A final concentration of 1 unit/ml of yeast pyrophosphatase (Fermentas) and 300 unit/ml phi29 DNA polymerase (Fermentas) was added into the composition described above to bring final volume to 50 µl. Reaction were incubated at 30°C for 5 h and terminated by heating to 65°C for 10 min. 3 µg of DNA products amplified from MDA reaction using p3KpadhET as templates was later digested with *Nde*I.

Transformation of strain BG1

Digested DNA products from MDA reaction was transformed into strain BG1 using electroporation as described previously [Tyurin *et al.* 2004]. Electropulsed cell suspensions were recovered and cultured in liquid BA medium with 5g/L glucose and later transferred into the same medium

supplemented with 50µg/ml kanamycin. The cultures grown with kanamycin were later transferred into roll tubes for single colony isolation. Primers used in PCR analysis of the transformant were listed below:

ldhcw1: 5'TAGCTACAGTAGGGGGTAGAGG 3'

lacGccw2: 5'AAAACTTGAGGTGCTTGGTGCTGC3'

adhEF1: 5'AAGGAGGACTGCATATGCCTACTTTATTACAAGAAAAAAAGG 3'

adhER1: 5' GAAATATATAGAAAAGCCTATGGAGAATAACTCGAGGG 3'

adhEF2: 5' CCACCAGCGGTACAGGTTCTG 3'

adhER2: 5' CCTGGCAATGTCCCCTGCTACATTG 3'

htkmidF: 5'CGATGCGATCTGTGCCCTTATCG 3'

htk3R: 5' CATTTTGAACGATGACCTC 3'

ldh5exF: 5'GCTGCTTTAAGTGTCTCAGGATCTCC 3'

ldh3exR: 5'CAGAAGCCGTTACAGTTGGTCC 3'

RNA extraction and cDNA synthesis

Cells were cultivated in 50 ml of BA media with 5g/L xylose as growth substrate at 70°C under anaerobic conditions. Cultures at an OD578 of ~0.5 were harvested by centrifugation at 5000 g and 4°C for 10 min. Total RNA extraction was performed with RNAqueous kit (Ambion, Applied Biosystems, Austin, USA) following the manufacturer's protocol. RNA integrity was observed after electrophoresis on 0.7% agarose gel and ethidium bromide staining. RNA was quantified by measuring absorbance at 260 nm. Measurement of the A260nm/A280nm ratio was used to assess the nucleic acid purity. 1 µg of total RNA was treated with 1 U of RNase-free DNase (Fermentas) for 1 h at 37°C, followed by inactivation of DNase at 65°C for 10 min in the presence of 2 mM EDTA. DNA-free RNA was reverse transcribed into cDNA using M-MuLV reverse transcriptase (Fermentas), as specified by the manufacturer.

Real-time quantitative PCR (RTQ-PCR)

Target gene (*adh*E) expression was reported according to the internal standard (*pgk*) expression in the same extract. The primers used in RTQ-PCR were *adhE*F (5'CCACCAGCGGTACAGGTTCTG3') and *adhE*R (5'CATGACA-ATTCCACCCTCTGTAACTG3'); *pgk*F (5'CAGGTGTACCTTATGAAGTA-GTGGATATAG3') and *pgk*R (5'GTAGTGTGGAACGGGCCTATGG3'). The RTQ-PCR reactions were performed in a 25 µl reaction volume containing 1X PCR buffer (Fermentas), 2.5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates (Fermentas), 600 nM (each) primer, 1 U of *Taq* polymerase (Fermentas), 1X

Sybr Green (Invitrogen A/S, Taastrup, Denmark) and 2 µl of 10X diluted cDNA product (water was used as non-template control). All PCR reactions were performed in triplicate and repeated twice. Sequence-specific standard curves were generated using eightfold serial dilutions (30 to $3*10^8$ copies) of pGapadhEK plasmid that containing both *pgk* gene and *adh*E gene. Reactions were run on an MiniOpticon System (Bio-Rad Laboratories, Symbion Science Park, Copenhagen, Denmark) using the following program: initial denaturation of 5 min at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 60°C, and 20 sec at 72°C; and a final elongation at 72°C for 7 min. Fluorescence was quantified on-line and at the end point with the sequence detection system software (Supports Opticon Monitor™ Version 3.1, Bio-Rad). Melting curve analysis was performed at 65-95°C with stepwise fluorescence acquisition. Data was analyzed and calculated based on the Opticon Monitor software. The specificity of the PCR reaction was verified by electrophoresis on 2% agarose gel and ethidium bromide staining.

Fermentation and analytical techniques

All fermentation experiments were performed as batch fermentations under strictly anaerobic conditions using 10% (v/v) inoculum. 26 ml serum tubes were used in the experiment with 10 ml of BA media supplemented with 5 g/L glucose or xylose. The triplicate cultures were inoculated with overnight culture and a sample after inoculation was saved for analysis. The cultures were grown at 70°C and the samples were collected after 48 h of growth. Cell density (OD) was measured at 578 nm in a spectrophotometer (Milton Roy Spectronic 301, Bie & Berntsen A-S, Denmark). Samples used for sugars and fermentation products determination were prepared and analyzed using HPLC as described previously [Georgieva *et al.* 2007].

4.4 Results

Chromosomal integration and isolation of strain BG1E1

Strain BG1 wild type transformed with linearized p3KpadhET was recovered with growth observed after 2 days in selective liquid medium. Presumptive chromosomal integrant isolates were picked from roll tubes containing 50µg/ml kanamycin. All picked isolates grew in liquid medium containing kanamycin and yielded identical results following PCR analysis (Data not shown). One of the isolate was chosen for further study and designated

strain BG1E1. Fig. 4.3 shows the map of chromosomal integration of *adh*E and *htk* gene in BG1E1 (B) compared with wild type strain (A).

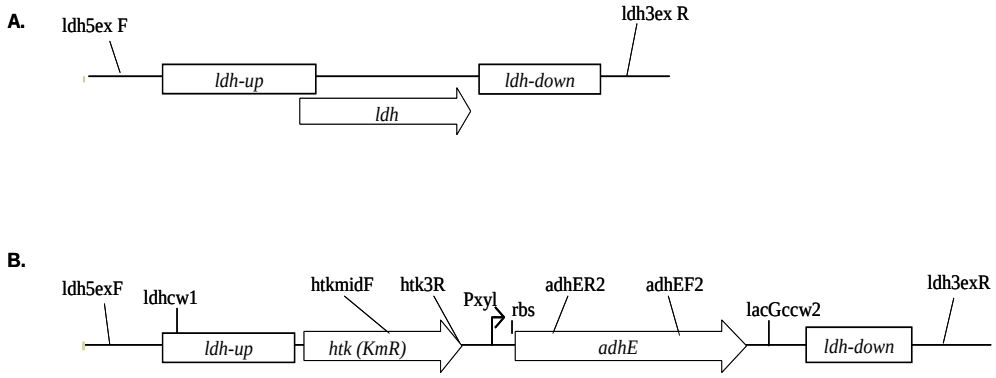

Figure 4.3 A. Strain BG1 wild type *ldh* gene and flanked upstream and downstream region; **B.** Map of double crossover region with *⊿ldh* and inserted *htk* and *adhE* gene in BG1E1 mutant strain.

Molecular characterization of strain BG1E1

Genomic DNA of strain BG1E1 was used as template in PCR amplification analysis with the primers shown in Fig. 4.3B. Three primer pairs of htkmidF - adhER2, ldhcw1 - htk3R, adhEF2 - lacGccw2 were used in PCR reactions and resulted in three fragments with the expected sizes of 1.25 kb, 1.45 kb and 1.02 kb, respectively (Fig. 4.4, lane 2, 4, 5), confirming the presence of the inserted *htk* gene and *adh*E gene. PCR was also conducted using primer pairs of adhEF2 - ldh3exR and yielded a 1.99 kb fragment (Fig. 4.4, lane 3), which is the expected size for chromosomal integration of *adh*E gene. Primer pair ldh5exF - htk3R yielded a 1.90 kb fragment (Fig 4.4, lane 1), which was the expected size for a double-crossover event (Fig 4.3B). All the above PCR products were sequenced and confirmed. Negative controls with wild-type genomic DNA using the same PCR mix were conducted and none of them showed any PCR product.

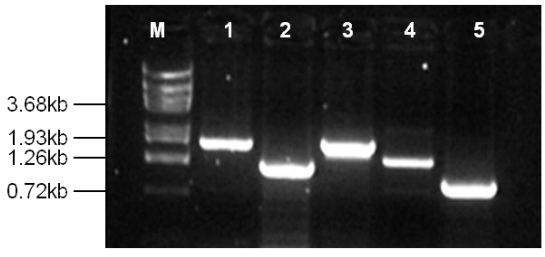

Figure 4.4 PCR analysis of BG1E1 mutant strain using genomic DNA as the template. Marker: Lambda DNA/Eco911 digested with *BstE*II (Fermentas); lane1: primers ldh5exF and htk3R, lane 2: primers htkmidF and adhER2; lane 3: primers adhEF2 and ldh3exR; lane 4: primers ldhcw1 and htk3R; lane 5: primers adhEF2 and lacGccw2

The expression of adhE in strain BG1E1

To study the *adh*E gene expression in BG1E1 strain, RTQ-PCR for the total cDNA of the growing log-phase cells was performed to investigate the transcriptional levels of *adh*E gene in different strains and medium conditions. The expression level of adhE was studied in strain BG1E1 in comparison with an ldh knockout strain, BG1L1, using either glucose or xylose as a substrate. The expression level of the target gene is based on adhE transcripts versus those of pgk (phosphoglycerate kinase), which encodes a constitutively expressed glycolytic enzyme in BG1. With glucose as a substrate, strains BG1L1 and BG1E1 showed a similar transcript level of adhE with an adhE/pgk transcript ratio of 2.73 and 1.86, respectively. In contrast, when xylose was used as a substrate, the expression level of adhE was clearly increased in strain BG1E1 as compared to BG1L1 with an adhE/pgk transcript ratio of 7.02 and 0.85, respectively. Therefore, the expression level of *adh*E in strain BG1E1 was confirmed to be up-regulated by the *Pxyl* promoter in the presence of xylose.

Effect of adhE overexpression on ethanol formation

Furthermore, the effect of induced adhE overexpression on ethanol formation was investigated in batch fermentation using different BG1 strains. Table 4.1 summarizes the results of the experiment. A comparison of the results using different genotypes shows the effect of induced adhE overexpression in BG1E1 strain and the effect of eliminating a lactate dehydrogenase from both BG1L1 and BG1E1 strains. Strains of BG1L1 and BG1E1 showed no detectable lactate production and higher ethanol yield as compared with the wild type on both of the tested substrates. With glucose as a substrate, strains of BG1L1 and BG1E1 showed similar metabolic patterns while strain BG1E1 showed a slightly lower ethanol yield as compared to strain BG1L1. When xylose was used as a substrate, an increased ethanol yield by 10% was observed with strain BG1E1 as compared with strain BG1L1. An ethanol yield of 1.59 mol/mol, corresponding to 95% of the maximal theoretical yield, was obtained with strain BG1E1 using xylose as a substrate.

Table 4.1 Product profile of different *T. mathranni* strains grown on different carbon sources

Strain	Glucose			Xylose		
	WT	BG1L1	BG1E1	WT	BG1L1	BG1E1
Sugar (mM)	28.3±0.9	28.8±2.5	26.8±0.4	34.9±2.5	35.3±0.7	35.9±1.1
Lactate (mM)	7.0±3.5	ND	ND	1.79±0.2	ND	ND
Acetate (mM)	4.1±0.9	6.0±0.4	8.1±1.0	4.8±0.3	6.1±1.1	3.9±0.6
Ethanol (mM)	45.5±1.8	50.8±4.5	43.0±2.8	49.4±3.0	51.2±1.0	57.0±1.1
$Y_{Lactate}$ [a]	0.25	0.00	0.00	0.05	0.00	0.00
$Y_{Acetate}$ [a]	0.14	0.21	0.30	0.14	0.17	0.11
$Y_{Ethanol}$ [a]	1.61	1.76	1.60	1.41	1.45	1.59
$T_{Ethanol}$ [b], %	80.5	88.0	80.0	84.4	86.8	95.2
CN [c] , %	100±1.7	98±0.1	95±1.9	96±4.2	97±1.5	101±2.3

Results of batch experiments using 5 g/L of the carbon source in BA medium after 48 h of incubation ; the values shown are averages of triplicate experiments +/- standard deviations. ND = Not detected.
[b.] $Y_{Lactate}$, $Y_{Acetate}$, $Y_{Ethanol}$, $Y_{Hydrogen}$ values are product yield based on mol/mol
[c.] Percentage of ethanol produced relative to the theoretical maximum
[d.] Carbon recovery

4.5 Discussion

Strain BG1E1, a recombinant strain of *T. mathranii* BG1 with a deletion of the *ldh* gene and an insertion of an *adh*E gene under the control of a regulated promoter, was constructed and characterized. Data from PCR and sequencing confirmed a homologous recombination-mediated knockout of the *ldh* gene, along with the chromosomal integration of a single copy *adh*E gene via a double crossover event in strain BG1E1. RTQ-PCR on total cDNA confirmed the up-regulation of *adh*E expression by xylose in strain BG1E1.

AdhE was conditionally expressed from a strong regulated promoter in a recombinant strain of *T. mathranii*, BG1E1, in which the expression level of adhE gene was up-regulated in the presence of xylose. Fermentation studies using different genotypes showed that the production of lactate was eliminated in both BG1L1 and BG1E1 strains, suggesting that the lactate formation pathway was inactivated by a stable *ldh* gene deletion. Fermentation studies using different carbon sources showed that the up-regulation of adhE expression level in strain BG1E1 with xylose as a substrate resulted in an increased ethanol yield as compared with its background strain BG1L1. Therefore, identification of the gene responsible for ethanol production provides a partial elucidation of the ethanol metabolism pathway in *T. mathranii* and facilitates the construction of an improved strain with a high ethanol yield by genetic engineering. Although the regulated expression level of AdhE is informative, strains in which the AdhE is constitutively expressed should be constructed to facilitate the ethanol production from all the sugars present in the lignocellulosic biomass including glucose.

4.6 References

Bryant MP. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. *Am J Clin Nutr.* 12:1324–1328.

Desai SG, Guerinot ML and Lynd LR. 2004. Cloning of L-lactate dehydrogenase and elimination of lactic acid production via gene knockout in *Thermoanaerobacterium saccharolyticum* JW/SLYS48 5. *Appl Microbiol Biotech* 65, 600-605

Georgieva T, Mikkelsen MJ and Ahring BK. 2007. Ethanol production from wet-exploded wheat straw hydrolysate by thermophili anaerobic bacterium *Thermoanaerobacter* BG1L1 in a continuous immobilized reactor. *Appl Biochem Biotech* 145,99-110

Hungate RE. 1969. A roll tube method for the cultivation of strict anaerobes. *Methods Microbiol* 3B, 117-132.

Larsen L, Nielsen P and Ahring BK. 1997. *Thermoanaerobacter mathranii* sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland. *Arch. Microbiol.* 168, 114-119.

Membrillo-Hernandez J, Echave P, Cabiscol E, Tamarit J, Ros J and Lin ECC. 2000 Evolution of the adhE gene product of *Escherichia coli* from a functional reductase to a dehydrogenase. *J Biol Chem* 275, 33869-33875

Mikkelsen MJ, Ahring BK. 2007. World intellectual property organization, WO/2007/134607, *Thermoanaerobacter Mathranii* strain BG1

Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Tyurin MV, Desai SG and Lynd LR. 2004. Electrotransformation of *Clostridium thermocellum*. *Appl Environ. Microbiol* 70, 883-890.

Chapter 5

Effect of expressing an NAD^+ dependent glycerol dehydrogenase on ethanol production in *Thermoanaerobacter mathranii*

5.1 Abstract A genetically engineered *Thermoanaerobactor mathranii* BG1 strain has been constructed to meet the NADH requirements for increased yield of ethanol from xylose by strategically implementing metabolic pathway. A recombinant strain, BG1G1, was created by expressing a heterologous NAD^+ dependent glycerol dehydrogenase (GLDH) and deleting the *ldh* gene encoding lactate dehydrogenase. The *gldh* gene placed under the control of P_{xyl} promoter was expressed in the presence of xylose, as detected by NAD^+ dependent GLDH activity. The resulting BG1G1 strain showed no lactate production and higher ethanol yield than wild type on both glucose and xylose. With GLDH induced NADH production, an increase of carbon flow to ethanol was observed in strain BG1G1 with higher ethanol yield (1.57 mol mol^{-1} xylose) and higher ethanol to acetate ratio (Et/Ac=14.86) than *ldh* deficient strain (BG1L1). Under a more reduced environment, increased ethanol yield was coupled with considerable less hydrogen production in strain BG1G1. The amount of NADH produced from GLDH could be controlled and optimized in favour of ethanol production pathway by supplementing a varying amount of glycerol. The possibility of constructing the novel nonnatural glycerol metabolic pathway in strain BG1G1 was discussed and demonstrated as a desired ethanol producer to meet both NADH requirement and carbon demand to produce high quantities and yield of ethanol.

5.2 Introduction

Metabolic engineering, the deliberate alteration of an organism's metabolism through genetic manipulation [Cameron, 1993], often involves amplification or introduction of a naturally occurring metabolic pathway. The full potential of metabolic engineering in the longer term include the creation of new metabolic pathways using enzymes, or modified enzymes, in functions different from those for which they evolved. Metabolic engineering of end-product metabolism has been pursued extensively in *Escherichia coli*, resulting in strains of industrial interest that produce high yields of ethanol as well as other products [Desai *et al.* 2004]. Both Gram-positive and thermophilic bacteria have unique advantages compared to Gram-negative bacteria [Dien *et al.* 2003]. However, such metabolic engineering has been pursued only to a limited extent in Gram-positive, mesophilic, obligate anaerobes, and Gram-positive obligate anaerobic thermophiles [Desai *et al.* 2004].

Thermoanaerobacter mathranii strain BG1 is a thermophilic, anaerobic, Gram-positive bacterium originally isolated from an Icelandic hot spring [Mikkelsen and Ahring 2007]. This organism ferments glucose, xylose, arabinose, galactose and mannose simultaneously and produce ethanol, acetate, lactate, CO_2, and H_2 as fermentation end products. Strain BG1 is of great interest because of its broad carbohydrate utilization range, high temperature growth optimum and its potential use for produciton of ethanol from biomass [Mikkelsen and Ahring 2007]. In our study of strain BG1, genetic modification tools had been developed and used for insertion, deletion or over-expression of the target genes. Furthermore, great efforts have been made on metabolic engineering of *T. mathranii* BG1 for improved ethanol production. The gene encoding for lactate dehydrogenase (*ldh*) has been deleted, resulting in a significantly improved ethanol producing mutant strain- BG1L1 [Georgieva *et al.* 2007]. The construction of 4 different alcohol dehydrogenase (*adh*) deficient mutant strains help to identify an AdhE isozyme responsible for ethanol production in *T. mathranii* BG1 [Chapter 3]. Further overexpression of AdhE and deletion of *ldh* gene in a recombinant BG1 strain (BG1E1) resulted in an increased ethanol yield [Chapter 4].

During catabolism of *T. mathranii* BG1 (Fig. 5.1), the cell utilizes carbon sources, such as xylose, by the pentose phosphate pathway. The metabolic flux of the pentose phosphate pathway is channelled into the upper and middle part of the Embden-Meyerhof pathway as fructose 6-phosphate and glyceraldehyde

3-phosphate, repectively [Zubay 1988]. Fructose 6-phosphate and glyceraldehydes 3-phosphate are, during catabolism, converted via pyruvate to different fermentation products. Ferredoxin is present in thermophilic anaerobic bacteria [Lynd 1989]. The reduction and oxidation of this molecule is achieved by decarboxylation of pyruvate to acetyl-CoA, production of H_2, or generation of reducing equivalents in the form of NAD(P)H. NADH and NADPH ferredoxin oxidoreductases catalyse the production of NADH and NADPH, respectively. Hydrogenase (H_2-ferredoxin oxidoreductase) forms hydrogen, and when reducing equivalent was transferred to ferredoxin and released as hydrogen, pyruvate may be metabolized to acetate with the generation of additional ATP. If H_2 cannot be evolved, or H_2 production is limited, then NAD^+ could be regenerated to form reduced product – ethanol and lactate (Fig. 5.1). With cofactor-dependent ethanol production systems in *T. mathranii* BG1, cofactor manipulation may become crucial in order to increase system productivity because of the alternative pathways competing for NADH oxidation by lactate and hydrogen production.

In this study, we heterologously overexpress a biologically active NAD^+-dependent glycerol dehydrogenase (GLDH) from *Thermotoga maritima* [Huber 1986] in strain BG1. Genetic manipulation is based on integrating the *gldh* gene under the P_{xyl} promoter into the chromosome of strain BG1 by knocking out its native *ldh* gene. The *gldh* gene was regulated by the P_{xyl} promoter to start the transcription when xylose is present. The primary purpose of this paper is to investigate a genetic means of regenerating intracellular NADH in strain BG1 and study its effect on ethanol production. In the presence of newly expressed GLDH and glycerol, one mole of NADH will be formed when one mol of glycerol is converted to one mol of glycerone. The system allows the cells to obtain extra reducing power that otherwise will not be obtained by the utilization of the carbon of glucose and xylose (Fig. 5.1). Furthermore, glycerol was not used as fermentable carbon source for BG1 wild type strain, and the possibility of constructing a nonnatural glycerol metabolic pathway through expression of the NAD^+-dependent GLDH in *T. mathranii* BG1 is later discussed.

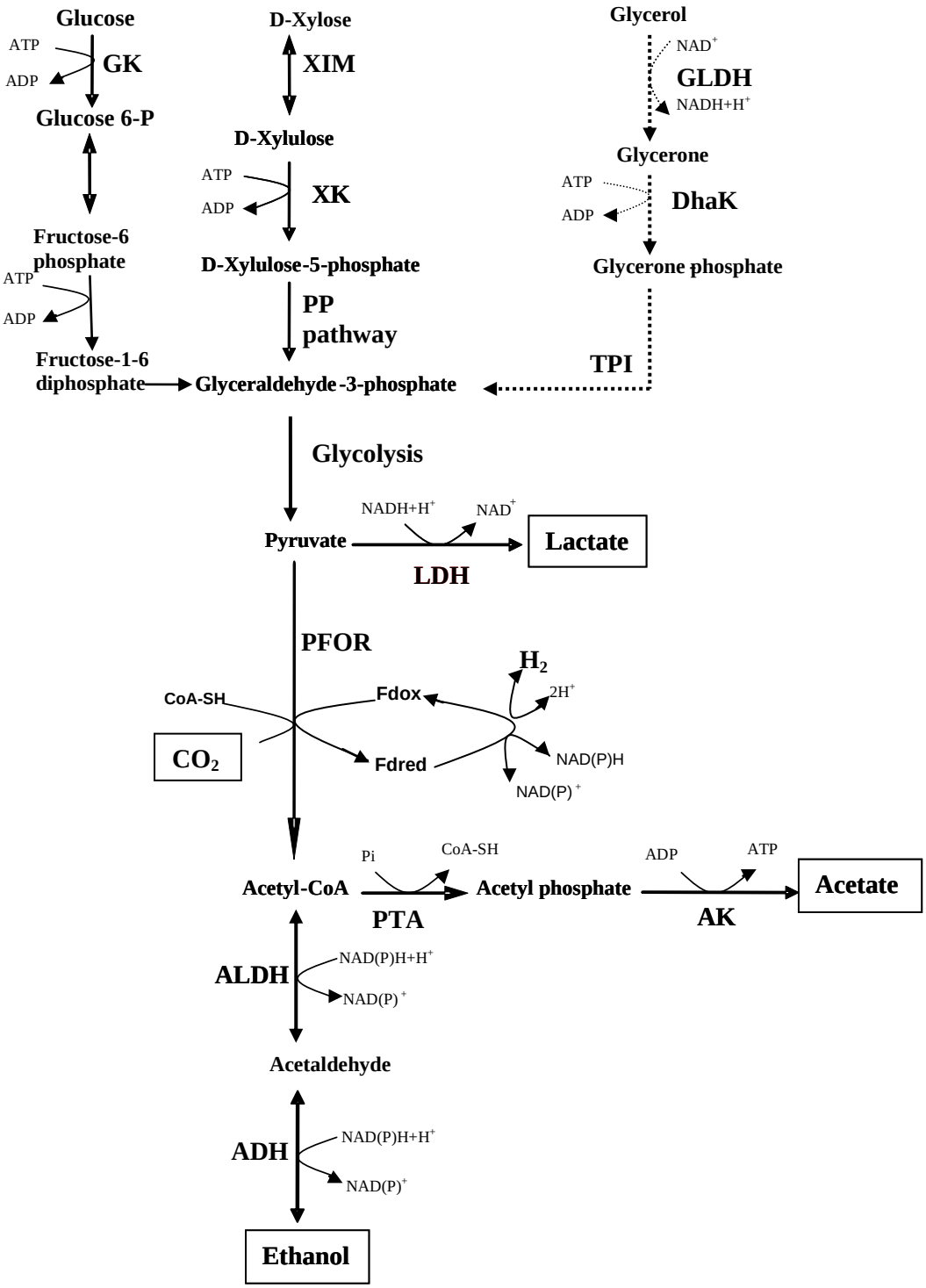

Figure 5.1 Model of anaerobic metabolism in thermophilic anaerobic ethanol producing bacteria and diagram illustrating the native cofactor independent upper part of glycolysis pathway and the newly introduced NAD^+ dependent glycerol degradation pathway. — Original NAD^+ independent pathway, ------ Newly added NAD^+ dependent pathway, GK=Glucokinase, XIM= Xylose isomerase, XK=Xylose kinase, PP pathway= pentose phosphate pathway, GLDH= Glycerol dehydrogenase, DhaK= Dihydroxyacetone kinase, TPI=Triosephosphate isomerase. LDH= Lactate dehydrogenase, PFOR= Pyruvate-ferredoxin oxidoreductase, PTA:=Phosphotransacetylase, AK= Acetate kinase, ALDH= Acetaldehyde dehydrogenase, PDC=Pyruvate decarboxylase, ADH= Alcohol dehydrogenase

5.3 Materials and methods

Culture origin, maintenance and cultivation

Strain BG1 was originally isolated from an Icelandic hot spring as previously described [Sonne-Hansen *et al.* 1993]. The strain was isolated and cultured anaerobically at 70°C in anaerobic synthetic BA medium as previously described [Larsen *et al.* 1997]. The medium was further supplemented with 2 g/L yeast extract and 5 g/L xylose or glucose as the growth substrate. Single colony was isolated using the anaerobic roll-tube technique [Hungate 1969, Bryant 1972] with medium solidified with Phytagel and $MgCl_2$. Colonies were picked with sterile needle, cultured and stored in 25% glycerol and 75% growth medium at -80°C in 2-ml sealed vials under 80%N_2/20%CO_2 atmosphere. Culture recovered from glycerol stocks were grown in liquid medium prior to use in experiments. For selection of antibiotic-resistant strains, the medium was supplemented with 50 μg/ml kanamycin and the culture was maintained anaerobically at 70°C.

Plasmids and PCR primers

Plasmid p3KpT was used as the source of the *ldh* deletion vector conferring thermostable kanamycin resistance. PCR primers were synthesized by MWG (MWG-BIOTECH AG, Ebersberg, Germany) and designed based on internal or external sequences of *ldh* gene, *gldh* gene and *htk* marker gene. Primers used in this work were:

ldhcw1: 5'TAGCTACAGTAGGGGGTAGAGG 3'
ldhccw2: 5'GTTTCAGGGCTGGCTGCGGCAAT 3'
gldhF1: 5'GCGGCGAGGAGGATAATTCATGATAACAACCACCATATTTCCAGG 3'
gldhR1: 5'CGGAAGGATGAGAAAAAACCTCACTTGACGCCGC 3'
gldhR2: 5'CAGATCTTTGGTGGAGAGTGTTCAG 3'
gldhF2: 5'GTGGGATTACCCACTACTCTGGC 3'
htkmidF: 5'CGATGCGATCTGTGCCCTTATCG 3'
htkdelR: 5'CTGTCAACAGAGGGAGTAGAGTTCAG 3'
ldhOUT F: 5' GAGCTGCTTTAAGTGTCTCAGG 3'
ldhOUT R: 5' CAGAAGCCGTTACAGTTGGTCC 3'

DNA preparation and manipulation

DNA fragments were subcloned using standard protocols [Sambrook and Russell 2001]. *E. coli* Top10 (Invitrogen A/S, Taastrup, Denmark)

transformants were grown in Luria-Bertani medium supplemented with ampicillin or kanamycin. Plasmid preparation and manipulation, genomic DNA preparation (A&A biotechnology, Poland) and transformation were carried out using standard procedures or the suppliers' instructions. Restriction enzymes were obtained from Fermentas (Fermentas GmbH, Germany) and New England Biolabs (Ipswich, England). *Taq* and *pfu* polymerase were purchased from Fermentas and DNA sequencing were conducted at MWG.

Cloning of gldh gene

The constructed vector, 7.07 kb p3KpgldhT was based on (Fig. 5.2) plasmid p3KpT. 1.12 kb *gldh* gene with ribosome binding site (rbs) was amplified from *T. maritima* genomic DNA using gldhF1 and gldhR1 primers. PCR amplification was performed with *Taq* and *pfu* polymerase. The resulting PCR fragments were treated with T4 polynucleotide kinase (Fermentas) and cloned into *Sma*I digested p3KpT plasmid after treatment with CIP (Calf Intestinal Phosphatase) from Fermentas. Following standard subcloning procedure, the constructed plasmid of p3KpgldhT was obtained. Restriction analysis and DNA sequencing confirmed the presence and orientation of the cloned *gldh* gene, *htk* marker gene as well as the flanking regions.

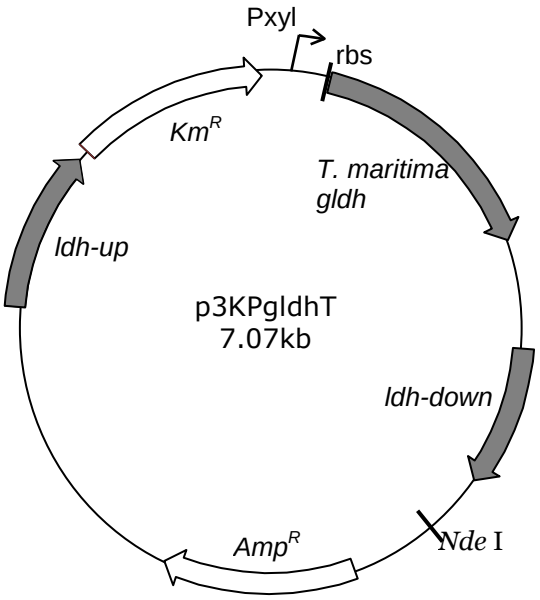

Figure 5.2 Construction of pUC19 based vector p3KpgldhT. *Km*, kanamycin marker gene; *Amp*, ampicillin marker gene; *ldh-up* and *ldh-down*, *ldh* upstream and downstream DNA fragments; *gldh*, glycerol dehydrogenase gene.

Multiple displacement amplification (MDA) and enzyme digestion

p3KpgldhT plasmid DNA (10ng-100ng) was placed into 0.2 ml PCR tubes in a total volume of 48 µl containing 33 mM Tris-acetate (pH 7.9), 10 mM Mg-acetate, 66 mM K-acetate, 0.1% (v/v) Tween 20, 1 mM DTT, 0.4 mM dNTPs and 50 µM exonuclease-resistant hexamer (Fermentas). The mixture was heating treated at 95°C for 2 min and chilled to 4°C in PCR System Thermocycler. A final concentration of 1 unit/ml of yeast pyrophosphatase (Fermentas) and 300 unit/ml phi29 DNA polymerase (Fermentas) was added into the composition described above to bring final volume to 50 µl. Reaction were incubated at 30°C for 5 h and terminated by heating to 65°C for 10 min. 3 µg of amplified DNA products from MDA reaction using p3KpgldhT as templates was later digested with *Nde*I.

Transformation of strain BG1

Digested DNA products from the MDA reaction was transformed into strain BG1 using electroporation as described previously [Tyurin *et al.* 2004]. Electropulsed cell suspensions were recovered and cultured in liquid BA medium with 5 g/L glucose and later transferred into the same medium supplemented with 50 µg/ml kanamycin. The cultures grown with kanamycin were later transferred into roll tubes for single colony isolation.

GLDH assay

The GLDH activity of the tested strain was determined as described below. The tested strains were cultivated in 100 ml of BA media with 5 g/L glucose/xylose and 2.5 g/L glycerol as growth substrate at 70°C under anaerobic conditions. Cultures at an OD578 of ~0.5 were harvested by centrifugation of 50 ml of the culture at 40000 rpm and 4°C for 30 min. The pellet was resuspended in 2 ml of ice chilled extraction buffer composed of 50 mM Tris-HCL, 10% glycerol and 1 mM $MgCl_2$ at pH 8.0. The cells were sonicated for 2 min in an ice bath (Digital Sonifier: Model 250; Branson Ultrasonics Corporation, Danbury, U.S.A.). The sonicated cells were centrifuged at 20000 g and 4°C for 30 min. The supernatant was used for GLDH activity assay at 70°C and pH 8.0 using the continuous spectrophotometric rate determination methods as previously described [Burton 1955]. One unit was defined as the amount of enzyme that produced 1 µmol of NADH per minute at 70°C and pH 8.0. Total concentration in the cell extracts was routinely measured by the

Bradford method [Bradford 1976] using bovine serum albumin (BSA) as a standard.

Fermentation

All fermentation experiments were performed as batch fermentations under strictly anaerobic conditions using 10% (v/v) inoculum. 26 ml serum tubes were used in the experiment with 10 ml of BA medium supplemented with 5 g/L glucose/xylose and 2.5 g/L glycerol. The triplicate cultures were inoculated with overnight culture and a sample after inoculation was saved for analysis. The cultures were grown at 70^{o}C and the samples were collected after 48 h of growth. To measure the growth rate of the tested strain, cell density at OD578nm was measured every 60 min during the exponential growth phase. In glycerol gradient experiment, cultures were grown in 5g/L xylose supplemented with 0 g/L, 1 g/L, 1.5 g/L, 2.5 g/L, 3.5 g/L, 6.5 g/L and 12 g/L glycerol, respectively.

Analytical techniques

Cell density (OD) was measured at 578 nm in a spectrophotometer (Milton Roy Spectronic 301, Bie & Berntsen A-S, Denmark). Samples used for sugars and fermentation products determination were prepared and analyzed using HPLC as described previously [Georgieva *et al.* 2007]. H_2 was determined by gas chromatography. The gas chromatograph (GC Microlab, Århus, Denmark) was equipped with a thermal conductivity detector. Gases were separated by a molecular sieve (0.5 nm). The oven and injection port were at 110^{o}C; the detector was at 110^{o}C. The carrier gas was N_2 at a flow rate of 30 ml/min.

5.4 Results

Chromosomal integration and isolation of strain BG1G1

Strain BG1 wild type was transformed with linearized p3KpgldhT vector and transformants were recovered after 2 days in selective liquid medium. 5 presumptive chromosomal integrant isolates were picked from roll tubes containing 50 μg/ml kanamycin. All picked isolates grew in liquid medium containing kanamycin and yielded identical results following PCR analysis (Data not shown). In addition, none of the isolates produced detectable lactate. One of the isolates was chosen for further study and designated strain BG1G1. Fig. 5.3

shows the map of chromosomal integration of *gldh* and *htk* gene by knocking out the *ldh* gene in strain BG1G1 (B) compared with the wild type strain (A).

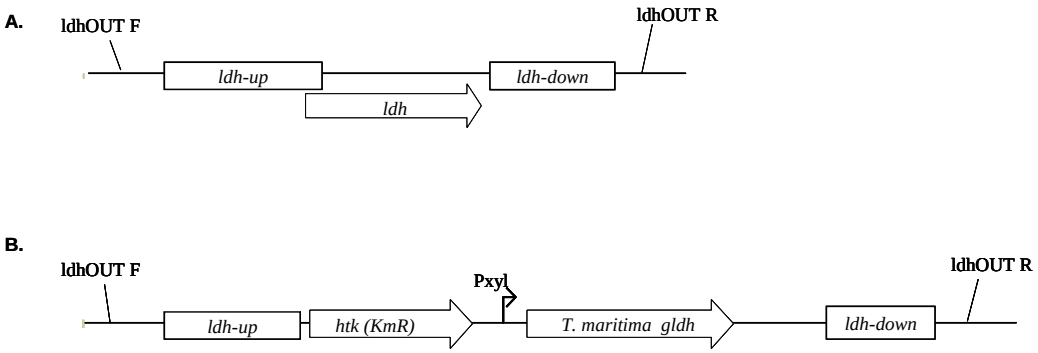

Figure 5.3 A. Strain BG1 *ldh* gene and flanked upstream, downstream region; **B.** Map of double crossover region with *Δldh* and inserted *htk* and *gldh* gene in strain BG1G1.

Molecular characterization of strain BG1G1

Gene disruption in strain BG1G1 was verified by PCR using genomic DNA as template compared with BG1 wild type and BG1L1 strains. The primer pairs, ldhOUTF - ldh3OUTR, located outside of the *ldh* region (Fig 5.3) were used in a PCR reaction and resulted in three fragments with the expected sizes of approximately 2.7 kb (wild type), 3.5 kb (BG1L1) and 4.6 kb (BG1G1), respectively (Fig 5.4A). All the amplified PCR fragments were further analyzed by enzyme digestion using restriction enzymes of *Eco*RI and *Pst*I, as shown in Fig.4B. The digested pattern of strain BG1G1 with both enzymes resulted in extra bands with the expected sizes compared with the control strains. In addition, all the PCR products were sequenced and confirmed the replacement of the *gldh* ORF with the *htk* marker cassette via a double crossover event (Fig. 5.3B) in strain BG1G1.

Enzymatic characterization of strain BG1G1

To confirm the integration of the *gldh* gene in strain BG1G1, the strain was also characterized by determining the GLDH activity. Table 1 shows the specific NAD^+ dependent GLDH activity of BG1 wild type, BG1L1 and BG1G1 strains in U/mg of total protein. One unit is defined as the amount of enzyme that produced 1 µmol of NADH per minute at 70°C and pH 8.0. A specific GLDH activity of 0.438 ± 0.04 U/mg was seen in strain BG1G1 grown on xylose and no activity was detected in strain BG1G1 grown on glucose. Wild type and BG1L1 strains showed no detectable GLDH activity on either of the substrates tested.

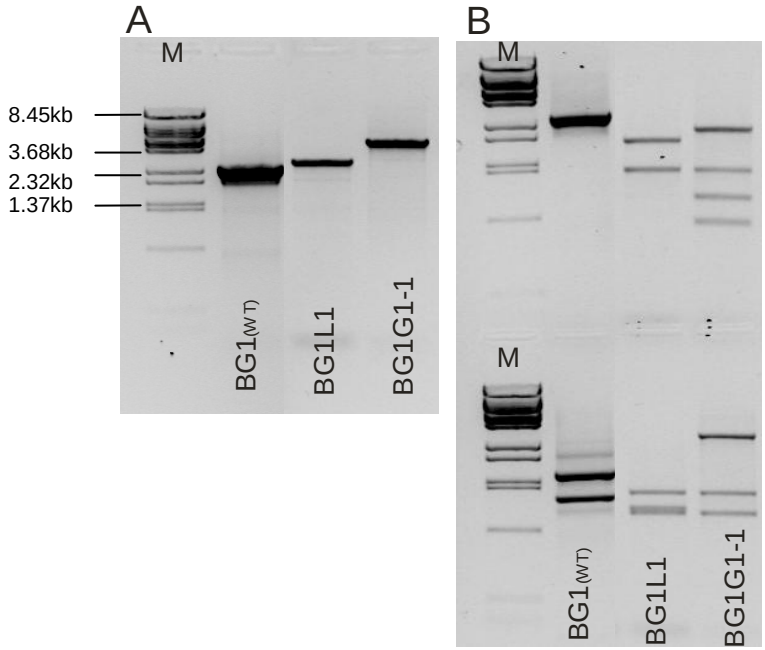

Figure 5.4 Verification of strain BG1G1 chromosome integration: **A**. PCR on chromosomal DNA from BG1 (wild type), BG1L1, and BG1G1 strains using external lactate dehydrogenase region primers (ldhOUTF - ldh3OUTR) **B**. Restriction analysis of the fragments shown in **A** using restriction enzymes *Eco*RI (upper part) and *Pst*I (lower part).

Table 5.1 Specific NAD^+ dependent Gldh activity of BG1 wild type, BG1L1 and BG1G1

Strain	Activity (U/mg)	
	Glucose	Xylose
BG1 wild type	ND	ND
BG1L1	ND	ND
BG1G1	ND	0.438 ± 0.04

Note. One unit is defined as the amount of enzyme that produced 1µmol of NADH per minute at 70 ^oC and pH8.0. Values shown are average of triplicates from anaerobic cultures. ND: not detected (less than 0.001U/mg)

Physiological characterization of strain BG1G1

Batch experiments in anaerobic tube were performed to investigate the metabolic pattern of BG1 wild type, BG1L1 and BG1G1 strains with different genotypic background. Table 5.2 shows the results of these experiments, including substrate consumption, fermentation product formation, ethanol to acetate ratio, product yields and carbon recovery after 48 h of growth. When grown on glucose or xylose, lactate was below the limit of detection for both BG1L1 and BG1G1 strains, but was readily detected for the wild type strain. The

level of metabolic products from strain BG1L1 was very similar to strain BG1G1 on glucose. On xylose, an increase of ethanol yield together with an increase of ethanol to acetate ratio (Et/Ac) was observed for strain BG1G1, as compared to both BG1L1 and wild type strains. Hydrogen was measured for the tested strains at the end of each of the fermentations. BG1G1 and BG1L1 strains were found to produce nearly the same amount of hydrogen on glucose while wild type produced slightly less hydrogen. When growing on xylose, strain BG1G1 produced much less hydrogen compared with the other two strains. Carbon recoveries were rather similar for the tested strains with values around 100%. Since glycerol was consumed by strain BG1G1, table 5.2 also shows the values including the glycerol as an extra carbon source. The glycerol consumption accounted for around 4% of the final carbon recovery.

Table 5.2 End-product analysis of glucose and xylose fermentation by BG1 wild type, BG1L1 and BG1G1 strains.

	Glucose			Xylose		
Strain	BG1(WT)	BG1L1	BG1G1	BG1 (WT)	BG1L1	BG1G1
Sugar (S) (mM)	25.4 ± 0.13	25.7 ± 2.24	22.9 ± 0.19	30.2 ± 1.56	30.1 ± 1.65	31.2 ± 1.94 [(a.) 2.06 ± 0.20]
Lactate (L) (mM)	9.2 ± 1.33	ND	ND	2.8 ± 0.30	ND	ND
Acetate (A) (mM)	6.2 ± 1.30	6.4 ± 0.60	5.6 ± 0.73	10.2 ± 0.87	7.4 ± 0.16	3.3 ± 0.45
Ethanol (E) (mM)	33.4 ± 1.33	43.6 ± 1.03	38.5 ± 0.53	34.3 ± 1.22	40.0 ± 2.04	49.0 ± 2.60
Hydrogen (mM)	13.4 ± 1.19	19.4 ± 3.81	18.01 ± 1.09	11.7 ± 0.60	18.5 ± 1.01	5.8 ± 0.86
Et/Ac ratio	5.355	6.808	6.863	3.384	5.406	14.859
$Y_{L/S}$	0.361	-	-	0.09	-	-
$Y_{A/S}$	0.245	0.249	0.245	0.336	0.246	0.106
$Y_{E/S}$	1.314	1.696	1.680	1.136	1.328	1.574
Carbon recovery (%)	96 ± 2.07	97 ± 0.77	96 ± 1.85	94 ± 3.01	95 ± 0.91	101 ± 1.30 [(b.) 97 ± 1.17]

Results are from anaerobic experiments using 5g/L glucose or xylose supplemented with 2.5g/L glycerol in BA medium after 48 h of culture. Values shown are of triplicate cultures. ND-Not detected (a): Glycerol consumption (b): carbon recovery calculated based on xylose and glycerol consumption.

Fig. 5.5 presents representative batch growth curves for BG1 wild type and BG1L1, BG1G1 mutant strains grown on xylose. Two other replicate experiments showed similar trends (data not shown). The time required to achieve substrate exhaustion and the final optical densities achieved in stationary phase were similar for the wild type and BG1L1 strains, but longer for BG1G1 strain. The specific growth rate calculated during exponential phase was 0.071 ± 0.001 for wild type, 0.072 ± 0.001 for BG1L1 and 0.019 ± 0.002 for BG1G1. Final optical density at the end of exponential phase was 0.93 ± 0.04 for wild type, 0.81 ± 0.03 for BG1L1 and 0.42 ± 0.03 for BG1G1. Compared with wild type and BG1L1 strains, strain BG1G1 showed slower growth rate, lower cell density and longer fermentation time to deplete the substrate. However,

strain BG1G1 showed overall higher ethanol concentration and lower acetate concentration without any lactate production at the end of fermentation compared with the other two control strains.

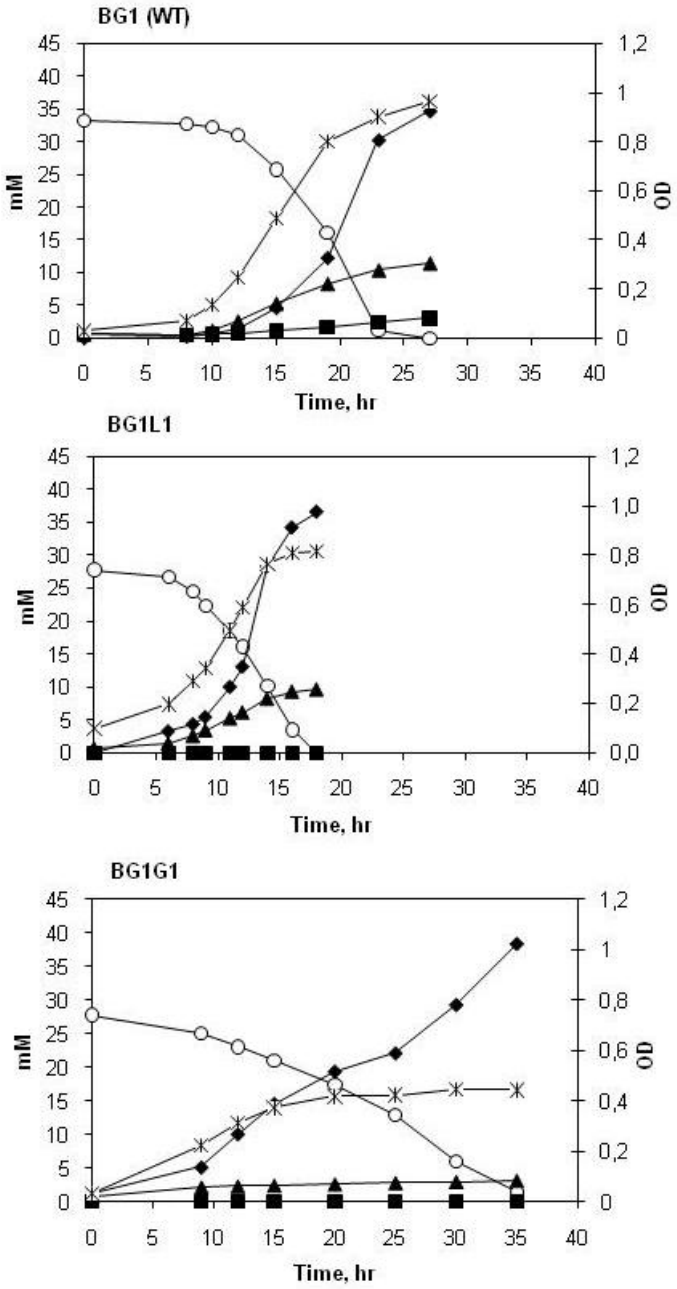

Figure 5.5 Growth curves for BG1 wild type strain and BG1L1, BG1G1 mutant strains on xylose. Substrate (o), lactate (■), acetate (▲), ethanol (♦) and OD (x)

In addition, the effect of supplementing the media with different levels of glycerol was also investigated in anaerobic tubes. Fig 5.6 shows the result of the experiment performed with strain BG1G1 in the media with different concentrations of initial glycerol supplement (0, 6, 10, 15, 29, 40, 70 mM). In the

absence of lactate production in BG1G1, Et/Ac was used to evaluate the optimal initial glycerol concentration in favour of ethanol production. Nearly all Et/Ac ratios increased with addition of glycerol in the media compared with the culture grown without glycerol and the highest Et/Ac was observed with glycerol supplementation at the initial concentration of 10 mM. Further increases of the initial glycerol concentration showed similar but slightly lower Et/Ac and a big decrease of Et/Ac was observed after the initial glycerol concentration was more than 40 mM.

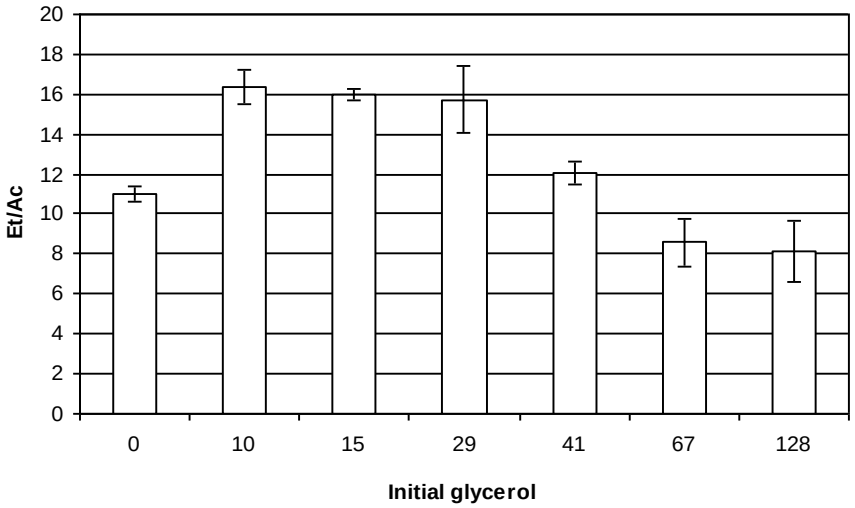

Figure 5.6 Results of anaerobic tube experiments with strain BG1G1 on 5g/ L xylose supplemented with different levels of glycerol. Values shown are averages of triplicate cultures.

5.5 Discussion

Strain BG1G1, a recombinant strain of *T. mathranii* BG1 featuring with deletion of *ldh* gene and insertion of foreign *gldh* gene under the control of P_{xyl} promoter, was constructed and characterized from different aspects. Data from PCR and sequencing confirmed a homologous recombination-mediated knockout of *ldh* along with the insertion of single copy *gldh* occurred via a double-crossover event. Enzymatic assay showed NAD^+ dependent GLDH activity only detected in strain BG1G1 with xylose as substrate, indicating the expression of GLDH in the strain and the expression is only induced when xylose is present.

Batch experiment in anaerobic tubes showed no detectable lactate production in strain BG1L1 and BG1G1 during growth on both glucose and xylose, suggest the effect of stable gene deletion. Elimination of lactate as a fermentation product resulted in proportionately increased yields of acetate

and ethanol in BG1L1 and BG1G1 strains without GLDH expression. But an increase of carbon flow to ethanol instead of acetate was observed with higher Et/Ac ratio when strain BG1G1 was grown on xylose with expressed GLDH activity. These results suggest that overexpression of the NAD^+ dependent GLDH may increase the intracellular NADH availability, and this in turn leads to a drastic shift in the metabolic pattern that favored the production of more reduced metabolite, ethanol, under a more reduced environment. It is evident that the cell adjusts its partitioning at the acetyl-CoA node by changing the ethanol (consume 2 NADH) to acetate (consumes no NADH) ratio to achieve a redox balance, as previously observed in experiments utilizing carbon sources with different oxidation states [San *et al.* 2002]. These findings also support the idea that NADH induces the expression of alcohol dehydrogenase (AdhE) [Leonardo *et al.* 1993], which catalyzes the production of ethanol during fermentation in *E. coli.* The same result was also seen in the metabolic engineering of *E. coli* by overexpressing an NAD^+ dependent formate dehydrgenase from *Canida boidinii*, the resulted mutant produced more ethanol with dramatic increase in Et/Ac ratio [Berrios-Rivera *et al.* 2002]. In addition, strain BG1G1 with the expressed GLDH activity produced considerably less H_2 with concomitant high ethanol yield suggest that the additional generated NADH is oxidized via production of ethanol rather than H_2, and this was associated with changes in activity of Fd-NAD oxidoreductases and alcohol-forming enzymes [Vasconcelos *et al.* 1994].

The growth curve for the tested strains on xylose again showed the ability of strain BG1G1 to produce overall higher concentration of ethanol and lower concentration of acetate without any lactate formation compared with wild type and BG1L1 strains. But this was accompanied by a slower growth rate, lower cell density and longer fermentation time to deplete the substrate for strain BG1G1. This negative effect on cell function, especially in the cell growth, has been widely observed upon enzyme overexpression. Overexpression of various glycolytic proteins in *Zymomonas mobilis* also had a negative effect on both glycolytic flux and growth rate [Snoep *et al.* 1995]. According to the proposed protein burden effect by Jacky *et al.*, this is possibly due to the competition of the overexpressed gene with the expression of all other genes or dilution effect of overexpressed enzyme over other enzymes [Snoep *et al.* 1995]. However, by overexpressing a cofactor dependent enzyme, the changes in both enzyme expression level and intracellular NADH concentration might have an influence

on the growth of the cells. In addition, the growth rate of BG1G1 strain could be improved by changing the final glycerol concentration in the medium in an immobilized cell reactor using BG1G1 for ethanol production (Mikkelsen *et al.*, data not shown).

Addition of glycerol to the media in anaerobic culture of BG1G1 strain showed a positive effect on ethanol production with higher Et/Ac ratio compared with the media without glycerol. The result suggests that it is necessary to supplement the culture with glycerol to activate the overexpressed NAD^+ dependent GLDH, which is used to produce NADH, and in turn leads to a change in metabolic pattern towards ethanol. The culture supplemented with glycerol at a final concentration of 10mM has the best stimulatory effect on the GLDH activity in favour of ethanol production with the highest Et/Ac ratio, further increase of the glycerol concentration did not have an effect or had a negative effect on the ethanol production with lower Et/Ac ratio. These results suggest that the amount of NADH in favour of ethanol production could be regulated and optimized by changing the GLDH activity with varying amount of glycerol.

Furthermore, two genes encoding dihydroxyacetone kinase (DhaK) and triosephosphate isomerase (TPI) involved in glycerolipid metabolism and glycolysis are present in *T. mathranii* BG1. In BG1G1 strain grown on xylose, the expression of GLDH catalyzes the oxidation of glycerol to glyceron (dihydroxyacetone), the intermediate that could possibly be directed to the glycolysis pathway by two sequential activities of DhaK and TPI (Fig 5.1). In this case, a novel pathway was engineered in *T. mathranii* BG1 to utilize glycerol as extra carbon source through the expression of the NAD^+-dependent GLDH. However, it is inconclusive by the batch experiments presented in this paper, but the presumption was later confirmed by ethanol fermentation using strain BG1G1 in an immobilized cell reactor (Mikkelsen *et al.*, data not shown).

This study demonstrates that metabolic engineering of *T. mathranii* BG1 by overexpressing an NAD^+ dependent glycerol dehydrogenase provide a more reduced environment and led to a shift towards the production of ethanol as the major fermentation product. Ethanol yield and growth rate of the resulting mutant strain (BG1G1) could be further optimized by changing the amount of supplemented glycerol in the media. Possibility of constructing the novel nonnatural glycerol metabolic pathway demonstrated a desired ethanol producer to meet both NADH requirement and carbon demand to produce high

quantities and yield of ethanol. This represents a step towards developing strains of thermophilic anaerobic bacteria that is double desirable in order to pursue advances in conversion of lignocellulosic biomass to ethanol.

5.6 References

Berrios-Rivera SJ, Bennett GN and San KY. 2002. Metabolic engineering of *Escherichia coli*: increase of NADH availability by overexpressing an $NAD^{(+)}$-dependent formate dehydrogenase. *Metab Eng* 4, 217-229.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biocehm* 72, 248-254.

Bryant MP. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria*. Am J Clin Nutr.* 25(12):1324–1328.

Burton RM. 1955. Methods in Enzymology, Volume I, 397

Desai SG, Guerinot ML and Lynd LR. 2004. Cloning of L-lactate dehydrogenase and elimination of lactic acid production via gene knockout in *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. *Appl Microbiol Biotech* 65, 600-605.

Georgieva T, Mikkelsen MJ and Ahring BK. 2007. Ethanol production from wet-exploded wheat straw hydrolysate by thermophili anaerobic bacterium *Thermoanaerobacter* BG1L1 in a continuous immobilized reactor. *Appl Biochem Biotechnol* 145, 99-110

Huber R, Langworthy TA, Konig H, Thomm M, Woese CR, Sleytr UB, Stetter KO. 1986. *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. *Arch.Microbiol*. 144, 324-333.

Hungate R E. 1969. A roll tube method for the cultivation of strict anaerobes. *Methods Microbiol* 3B, 117-132.

Larsen L, Nielsen P and Ahring BK. 1997. *Thermoanaerobacter mathranii* sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland. *Arch. Microbiol.* 168, 114-119.

Lee RL. 1989. Production of ethanol from lignocellulosic materials using thermophilic bacteria: critical evaluation of potential and review. *Adv Biochem Eng Biotechnol.* 38

Leonardo MR, Cunningham PR and Clark DP. 1993. Anaerobic regulation of the adhE gene, encoding the fermentative alcohol dehydrogenase of *Escherichia coli*. *J Bacteriol* 175, 870-878

Leonardo MR, Dailly Y, and Clark DP. 1993. Anaerobic engineering aspects of biocatalysis: Cofactor regeneration as example. *Biotech Bioeng* 52, 309-319

Mikkelsen MJ, Ahring BK. 2007. World intellectual property organization, WO/2007/134607, *Thermoanaerobacter Mathranii* strain BG1

Sambrook J, Russell DW, 2001, Molecular cloning: a laboratory manual Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

San KY, Bennett GN, Berrios-Rivera SJ, Vadali RV, Yang YT, Horton E, Rudolph FB, Sariyar B and Blackwood K. 2002. Metabolic engineering through cofactor manipulation and its effects on metabolic flux redistribution in *Escherichia coli*. *Metab Eng* 4, 182-192.

Snoep JL, Yomana LP, Westerhoff HV and Ingram LO. 1995. Protein burden in *Zymomonas mobilis*: negative flux and growth control due to overproduction of glycolytic enzymes. *Microbiol* 141, 2329-2337

Sonne-Hansen J, Mathrani IM, and Ahring BK. 1993. Xylanolytic anaerobic thermophiles from Icelandic hot-springs. *Appl Microbiol Biotechnol* 38:537-541

Tyurin MV, Desai SG and Lynd LR. 2004. Electrotransformation of *Clostridium thermocellum*. *Appl Environ Microbiol* 70, 883-890.

Vasconcelos I, Girbal L and Soucaille P. 1994. Regulation of carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. *J Bacteriol* 176, 1443-1450.

Zubay G.1988. Glycolysis, gluconeogenesis, and the pentose phosphate pathway. In:Biochemistry. Macmillan Publishing Company, Collier Macmillan Publishers, New York, London, pp436-480

Chapter 6

Effect of cofactor manipulation on ethanol production in *Thermoanaerobacter mathranii*

6.1 Abstract Cofactor manipulation in *Thermoanaerobacter mathranii* strain BG1 was achieved by both external and genetic strategies and the effect of these manipulations on ethanol production was assessed. Feeding the cells with carbon sources which have different oxidation states were used to address the NADH requirement for ethanol production in strain BG1. Mannitol generates one more mole of NADH than glucose or xylose. The use of mannitol as a carbon source was shown to increase the ethanol production in both BG1 wild type strain and *ldh* deficient strain than the use of the other two substrates, due to the increased NADH availability. *In vivo* system to manipulate the NADH level in strain BG1 was to express a heterologous glycerol dehydrogenase (GLDH) that can regenerate NADH or/and delete the native lactate dehydrogenase (LDH) to eliminating the NADH - competing pathway. With functional LDH and expressed GLDH, ethanol yield was significantly decreased accompanied by increased lactate formation, which was shown to be the preferred route for the regeneration of NAD^+. However, by inactivating the lactate formation pathway, the increased carbon flux was channelled toward acetyl-CoA and the cells adjusts its partitioning at the acetyl-CoA node to favour the production of ethanol to establish the redox balance under a more reduced environment. Therefore, *ldh* negative strain with increased NADH availability by genetic manipulation should render *T. mathranii* BG1 industrially interesting for ethanol production.

6.2 Introduction

Metabolic engineering has the potential to considerably improve process productivity by manipulating the flux of certain pathways. Applications of genetic engineering or metabolic engineering have increased in both academic and industrial institutions and the area has been reviewed [Lee and Papousakis 1999]. Most current metabolic engineering studies have mainly focused on manipulating enzyme levels through the amplification, addition, or deletion of a particular pathway. However, cofactors play an essential role in a large number of biochemical reactions and their manipulation has the potential to be used as an additional tool to achieve desired metabolic engineering goals.

Nicotinamide adenine dinucleotide (NAD) functions as a cofactor in over 300 oxidation–reduction reactions and regulates various enzymes and genetic processes [Berrios-Rivera *et al.* 2002, San *et al.* 2002]. The NADH/NAD^+ cofactor pair plays a major role in microbial catabolism, in which a carbon source, such as glucose, is oxidized using NAD^+ as cofactor and producing reducing equivalents in the form of NADH. It is crucially important for continued cell growth that NADH be oxidized to NAD^+ and a redox balance be achieved. Under aerobic growth, oxygen is used as the final electron acceptor. While under anaerobic growth, and in the absence of an alternate oxidizing agent, the regeneration of NAD^+ is achieved through fermentation, where NADH is used to reduce metabolic intermediates and to regenerate NAD^+ (Fig. 6.1). Therefore, in fermentation, alteration in the availability of NADH is expected to have a profound effect on the metabolite distribution.

The influence of cofactors in metabolic networks has been evidenced by studies in which the NADH/ NAD^+ ratio is altered by feeding carbon sources with different oxidation states [Leonardo *et al.* 1996, San *et al.* 2002]; or by expressing an enzyme like NADH oxidase [Lopez de Felipe *et al.* 1998]. Biotransformation with whole cells is still the preferred method for the synthesis of most cofactor-dependents products because the fact that the cells can regenerates the cofactor naturally. However, the enzyme of interest has to compete for the required cofactor with a large number of other enzymes within the cell. In cofactor dependent production systems, after the enzyme of interest have been overexpressed, the availability of the required form of the cofactor can become limiting, making cofactor manipulation crucial for optimal production.

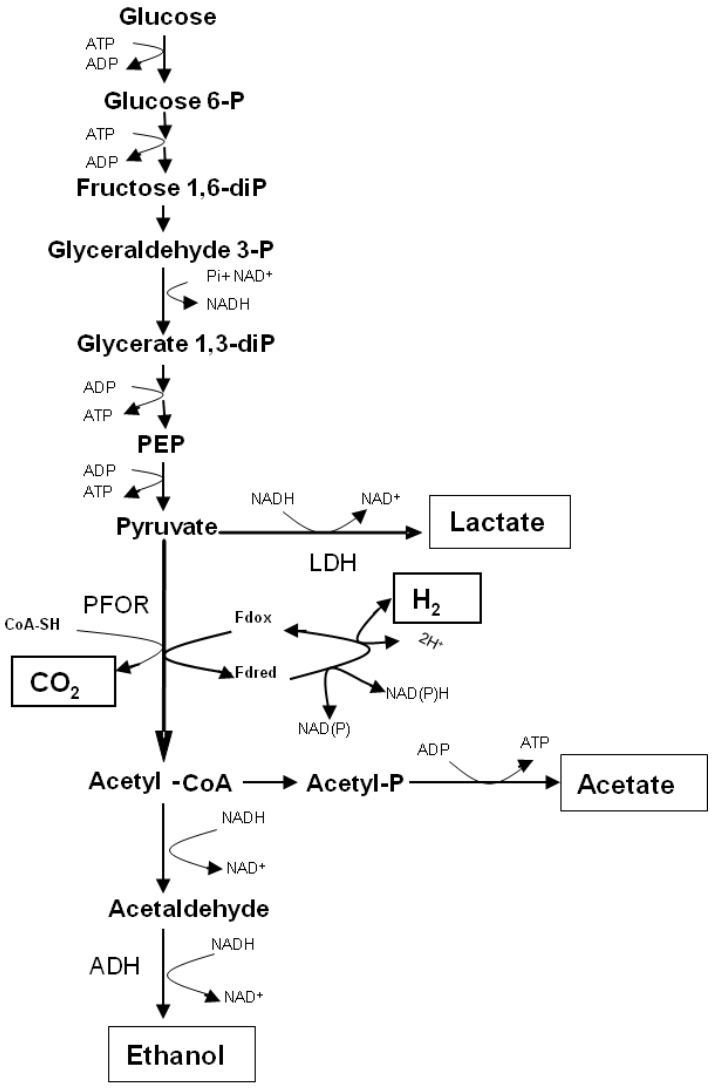

Figure 6.1 Model of anaerobic metabolism in thermophilic anaerobic ethanol producing bacteria showing NADH/NAD^+ and ATP/ADP. PEP, phosphoenolpyruvate. LDH, lactate dehydrogenase; PFOR, pyruvate ferredoxin oxidoreductase; ADH: alcohol dehydrogenase

Thermoanaerobacter mathranii strain BG1 is a thermophilic anaerobic bacterium that is of great interest because of its potential use for production of ethanol from biomass [Mikkelsen and Ahring *et al.* 2007]. Metabolic engineering of strain BG1 has been focused on addition, deletion or up/down regulation of particular pathway for the purpose of improved ethanol production [Chapter 3,4,5]. In *T. mathranii* BG1, the pathway of carbon metabolism are inextricably linked with cofactor oxidation and reduction reactions (Fig.6.1). Metabolism of glucose yields NADH from the glycolytic pathway and converted via pyruvate to the different end fermentation products. Pyruvate can be converted to either lactate with the oxidation of NADH or to acetyl-CoA and CO_2 with a concomitant reduction of ferredoxin (Fd_{red}). The re-oxidation of Fd_{red} could be catalyzed by different oxidoreductases.

NADH and NADPH ferredoxin oxidoreductases catalyse the production of NADH and NADPH, respectively. Hydrogenase (H_2-ferredoxin oxidoreductase) forms hydrogen, and when reducing equivalents are transferred to ferredoxin and released as hydrogen, pyruvate may be metabolized to acetate with the generation of additional ATP. If H_2 can not be evolved, or H_2 production is limited, then NAD^+ may be regenerated to form the reduced products – ethanol and lactate. The formation of the final end-fermentation products and their mutual distribution is determined by the response of the cells to a given metabolic state, their need for energy, metabolites for reductive biosynthesis and reducing equivalent [Zeikus *et al.* 1981].

Here we describe the conceptual designs of various approaches to manipulate the cofactors and study their effect on ethanol production in strain BG1. Two distinct strategies were used to alter the NADH availability. The first approach is based on the use of carbon source with different oxidation states. Fig. 6.2 highlights the differences in the oxidation state of the tested carbon source as they enter the glycolysic pathway. Mannitol produces more reducing equivalents in the form of NADH than glucose and xylose. The second strategy targets the genetic manipulation of the host cell. In this strategy, we try to manipulate the cofactor level by 1) overexpressing an enzyme that can regenerate NADH, 2) eliminating an NADH - competing pathway or 3) combining both genetic manipulation strategies. Intracellular NADH was regenerated through the heterologous expression of an NAD^+ dependent glycerol dehydrogenase (GLDH) from *Thermotoga maritima* in strain BG1. Elimination of the NADH competing pathway in strain BG1 was done by inactivation of the lactate formation pathway, which is coupled with NADH oxidation (Fig.6.1)

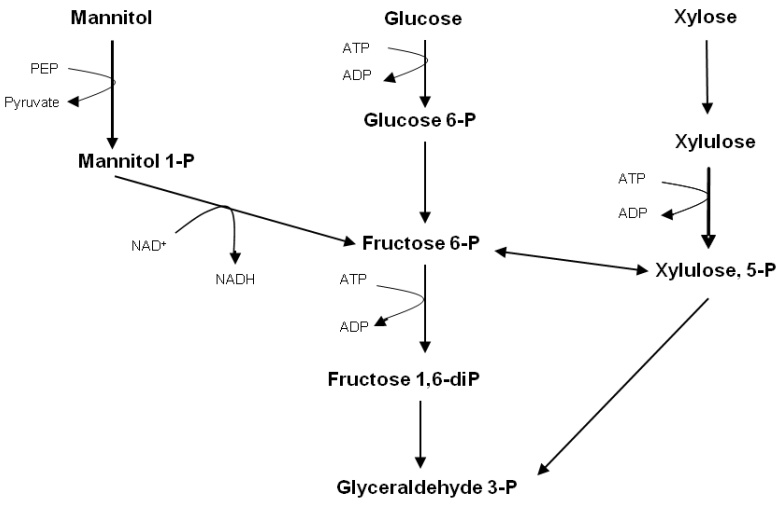

Figure 6.2 Schematic showing the differences in the oxidation of mannitol, glucose and xylose as they enter the glycolysis pathway.

6.3 Materials and methods

Culture origin, maintenance and cultivation

Strain BG1 was originally isolated from an Icelandic hot spring as previously described [Sonne-Hansen *et al.* 1993]. The strain was cultured anaerobically at 70°C in anaerobic synthetic BA medium as previously described [Larsen *et al.* 1997]. The medium was further supplemented with 2g/L yeast extract and 5g/L xylose or glucose as the growth substrate. Single colony was isolated using the anaerobic roll-tube technique [Hungate1969, Bryant 1972] with medium solidified with Phytagel and $MgCl_2$. Colonies were picked with sterile needle, cultured and stored in 25% glycerol and 75% growth medium at -80°C in 2-ml sealed vials under 80%N_2/20%CO_2 atmosphere. Culture recovered from glycerol stocks were grown in liquid medium prior to use in experiments.

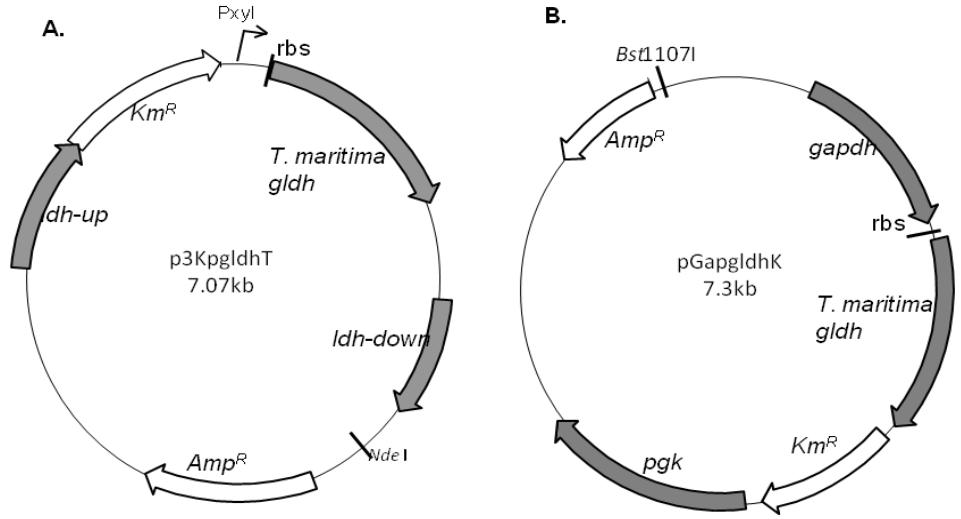

Figure 6.3 A. Construction of xylose controlled regulative expression vector p3KpgldhT; **B.** Construction of constitutive expression vector pGapgldhK. *Km*, kanamycin marker gene; *Amp*, ampicillin marker gene; *ldh-up* and *ldh-down*, *ldh* upstream and downstream DNA fragments; *gapdh*, truncated glyceraldehyde 3-phosphate dehydrogenase gene; *pgk*, phosphoglycerate kinase gene; *gldh*, glycerol dehydrogenase gene; *rbs*, ribosome binding site.

Strains and plasmids

Wild type BG1 strain was used for all genetic manipulations [Mikkelsen and Ahring 2007]. *T.* BG1L1 (*Δldh*) [Georgieva *et al.* 2007] is the mutant strain with the lactate pathway inactivated. Plasmid p3KpgldhT and pGapgldhK (Fig. 6.3 A. B) were used to overexpress an NAD^+ dependent glycerol dehydrogenase (GLDH) in strain BG1, resulting in mutant strains of BG1G1 (*Δldh*, P_{xyl}GLDH)

[Chapter 5] with inactivated lactate pathway and BG1G2 (GLDH) with functional lactate formation pathway.

GLDH assay

The GLDH activity of the tested strain was determined as described below. The tested strains were cultivated in 100 ml of BA media with 5 g/L glucose/xylose and 2.5 g/L glycerol as growth substrate at 70°C under anaerobic conditions. Cultures at an OD578 of ~0.5 were harvested by centrifugation of 50 ml of the culture at 40000 rpm and 4°C for 30 min. The pellet was resuspended in 2 ml of ice chilled extraction buffer composed of 50 mM Tris-HCL, 10% glycerol and 1 mM $MgCl_2$ at pH 8.0. The cells were sonicated for 2 min in an ice bath (Digital Sonifier: Model 250; Branson Ultrasonics Corporation, Danbury, U.S.A.). The sonicated cells were centrifuged at 20000 g and 4°C for 30 min. The supernatant was used for GLDH activity assay at 70°C and pH 8.0 using continuous spectrophotometric rate determination method as previously described [Burton 1955]. One unit was defined as the amount of enzyme that produced 1 µmol of NADH per minute at 70°C and pH 8.0. Total concentration in the cell extracts was routinely measured by the Bradford method [Bradford 1976] using bovine serum albumin (BSA) as a standard.

Fermentation

All fermentation experiments were performed as batch fermentations under strictly anaerobic conditions using 10% (v/v) inoculum. The triplicate cultures were inoculated with overnight culture and a sample after inoculation was saved for analysis. The cultures were grown at 70°C and the samples were collected after 48 h of growth. In the carbon source comparison experiment, 26 ml serum tubes were used in the experiment with 10 ml of BA media supplemented with 5 g/L glucose or xylose or mannitol as the carbon source. In the genetic manipulation experiment, 26 ml serum tubes were used in the experiment with 10 ml of BA media supplemented with 5 g/L xylose and 2.5 g/L glycerol.

Analytical techniques

Samples used for sugars and fermentation products determination were prepared and analyzed using HPLC as described previously [Georgieva *et al.* 2007]. H_2 was determined by gas chromatography. The gas chromatograph (GC

Microlab, Århus, Denmark) was equipped with a thermal conductivity detector. Gases were separated by a molecular sieve (0.5 nm). The oven and injection port were at 110°C; the detector was at 110°C. The carrier gas was N_2 at a flow rate of 30 ml/min.

6.4 Results and Discussion

Effect of carbon sources

Satisfying the cofactor requirement of metabolic pathway is essential in facilitating product formation. To study the effect of increased NADH availability on ethanol production, mannitol was used as a carbon source for comparison to glucose and xylose. For 1 mol of mannitol consumed, 1 mole more NADH are formed compared with the use of glucose and xylose as substrate. Fig 6.2 highlights the differences in the oxidation state of the tested carbon source as they enter the glycolysis pathway.

Batch experiments using different carbon sources with different oxidation state were performed to investigate the influence of NADH availability on ethanol production in BG1 wild type and BG1L1 (*Δldh*) strains. Table 6.1 and fig 6.4 summarize the results of the experiments. When wild type was used, ethanol yields with mannitol were 22.6% and 9.1% higher than with glucose and xylose, respectively, based on carbon (Cmol) $Cmol^{-}$. For strain L1, ethanol yields with mannitol were 8.5% and 13.8% higher than with glucose and xylose, respectively. The result showed that mannitol was a more effective carbon source in ethanol production than glucose and xylose, when both wild type strain and L1 were examined. Meanwhile, it also showed the importance of NADH as a rate-limiting factor in the ethanol formation pathway in strain BG1. Accompanied by the increased ethanol production, the acetate yield decreased substantially with mannitol over the other two substrates, for both strains. This decrease could be attributed to more carbon flux being directed towards the ethanol formation pathway rather than the acetate formation at the Acetyl-CoA node. The production of more reduced metabolites was favoured, as evidenced by dramastic increases in the ethanol-to-acetate ratio (Et/Ac) (Table 6.1) and a shift towards the production of ethanol as the major fermentation product, for both strains. Formation of lactate also requires NADH, which becomes a competitive pathway for the reduced cofactor with the ethanol formation pathway. Deletion of *ldh* in L1 eliminates the lactate production by inactivating the lactate formation pathway. This leads to increases of both carbon flux and

cofactor availability in the ethanol formation pathway, as evidenced by the overall higher ethanol yields in L1 than wild type. Furthermore, both strains showed higher hydrogen production with mannitol over the other two substrates, suggesting that the additional generated NADH is also oxidized via H_2 production in addition to the production of ethanol. Overall, these results show the significant effect of mannitol in rendering reduced environments through the formation of extra NADH during its catabolism.

Table 6.1 The effect of carbon source on metabolite yield molar using BG1 wild type strain and BG1L1 mutant strain.

	Wild type			BG1L1		
Strain	Glucose	Xylose	Mannitol	Glucose	Xylose	Mannitol
$Y_{L/S}$ [(a)]	0.163	0.032	0.076	0.00	0.00	0.00
$Y_{A/S}$ [(b)]	0.039	0.067	0.011	0.069	0.088	0.020
$Y_{E/S}$ [(c)]	0.491	0.552	0.602	0.586	0.559	0.636
$Y_{H/S}$ [(d)]	0.138	0.182	0.377	0.179	0.208	0.428
Et/Ac[(e)]	12.44	8.286	56.60	8.437	6.334	32.45
CN (%) [(f)]	95.79	95.90	99.59	98.30	97.10	98.37
	(2.85)	(2.56)	(1.34)	(1.73)	(2.34)	(0.87)

Results are from anaerobic experiment using 5g/L of the carbon source in BA medium after 48 h of culture. Values shown are of triplicate cultures (a). Yield for lactate (Cmol/Cmol); (b). Yield for acetate (Cmol/Cmol); (c). Yield for ethanol (Cmol/Cmol); (d). Yield for hydrogen (Cmol/Cmol); (e). Ethanol to acetate ratio; (f) Carbon recovery

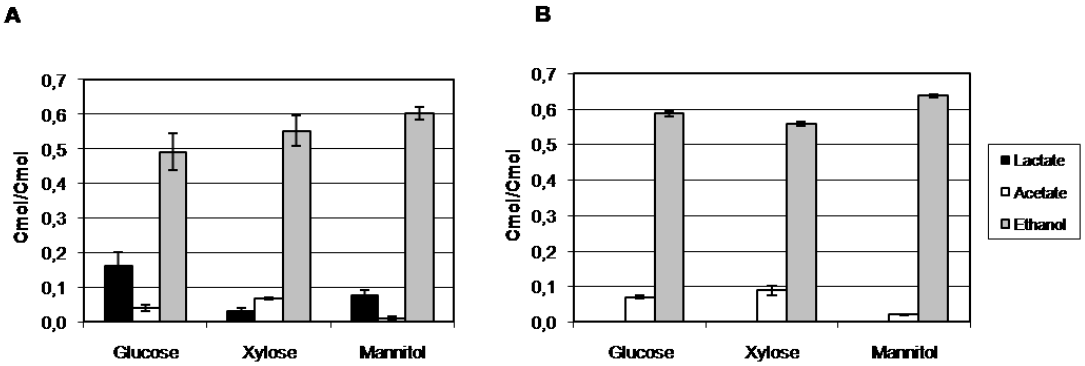

Figure 6.4 Products yield of BG1 wild type strain (A) and BG1L1 strain (B) grown on different carbon sources. Results are from anaerobic experiments using 5g/L carbon source in BA medium after 48 h of culture. The values shown are for triplicate experiments and the error bars correspond to the SD of the triplicates.

Effect of genetic manipulation

Genetic manipulations are based on direct cloning of glycerol dehydrogenase gene (*gldh*) derived from *Thermotoga maritima* into constructed vectors (Fig 6.3 A,B) and later transforming into strain BG1.

Construct A aims at express the *gldh* gene under the regulation of P_{xyl} promoter by knocking out its native lactate dehydrogenase (*ldh*) gene in strain BG1. Construct B aims to constitutively express the *gldh* gene by integration into the chromosome of strain BG1. Two mutant strains of BG1 were then created by transformation with these two different constructs, and named as strain BG1G1 (*Δldh*, P_{xyl}GLDH) and BG1G2 (GLDH), respectively. To confirm the GLDH expression resulting from the integration of *gldh* gene in BG1G1 and BG1G2 strains, they were characterized by determining the specific GLDH activity (Table 6.2). BG1 wild type and BG1L1 strains were used as control strains. The result showed that the expression of GLDH was introduced in strain BG1G1 grown on xylose and in strain BG1G2 grown on both substrates, as detected by NAD^+ dependent GLDH activity. The control strains, BG1 wild type and BG1L1 showed no detectable NAD^+ dependent GLDH activity. In the presence of the newly expressed GLDH and glycerol, one mol of NADH will be formed when one mol of glycerol is converted to one mol of glycerone by the activity of GLDH, thus allowing the cell to retain the extra reducing power that would otherwise not be obtained by utilizing the carbon sources like glucose and xylose. The formation of lactate also requires NADH in strain BG1 (Fig. 6.1). The effect of eliminating the lactate formation pathway that competes for NADH utilization on ethanol production in *Δldh* strain was also investigated.

Table 6.2 Specific NAD^+ dependent GLDH activity of BG1 wild type, BG1L1, BG1G1 and BG1G2 strains in U/mg of total protein

Strain	Activity (U/mg)	
	Glucose	Xylose
Wild type	ND	ND
BG1L1 (*Δldh*)	ND	ND
BG1G1 (*Δldh*, P_{xyl}GLDH)	ND	0.438 ± 0.04
BG1G2 (GLDH)	0.396 ± 0.02	0.287 ± 0.06

Note. One unit is defined as the amount of enzyme that produced 1 µmol of NADH per minute at 70 ^oC and pH 8.0. Values shown are average of triplicates from anaerobic cultures. ND: not detected (less than 0.001U/mg)

Batch experiments with BG1G1 and BG1G2 strains were performed to investigate the effect of genetic manipulation on ethanol production by using glucose or xylose supplemented with glycerol. Table 6.3 and fig 6.5 summarizes the results of the experiment. A comparison of the result with the control strain (wild type) indicates the effect of eliminating the native LDH in strain BG1L1 or

expressing a NAD^+ dependent GLDH in strain BG1G2, or the combined effect of both genetic manipulations in strain BG1G1. BG1L1 and BG1G1 strains showed no detectable lactate production during growth on both glucose and xylose, showing the effect of inactivating the lactate formation pathway by stable *ldh* gene deletion. Elimination of lactate as a fermentation product resulted in increased ethanol yields in BG1L1 and BG1G1 strains. However, with GLDH introduced NADH production in strain BG1G1 grown on xylose, a further increase of carbon flow to ethanol was observed. This is evidenced by 16.9% and 38.6% increased ethanol yield of BG1L1 and BG1G1 strains, respectively, relative to the wild type based on Cmol $Cmol^{-}$. Interestingly, constitutive *gldh* epxression in strain BG1G2 with a functional lactate formation pathway leads to a drastic shift in the metabolic pattern that favours the production of lactate instead of ethanol. This is different from results obtained previously from metabolic engineering of *E. coli* by expression of an NAD^+ dependent formate dehydrogenase from *Candida boidinii*. The resulting mutant showed a significant increased ethanol production accompanied by decreased lactate formation [Berrios-Rivera *et al.* 2002]. However, pyruvate ferredoxin oxidoreductase (PFOR) is present in *T. mathranii* BG1 and catalyzes the conversion of pyruvate to acetyl-CoA and CO_2 with concomitant reduction of ferredoxin. The re-oxidation of reduced ferredoxin can be catalyzed by different oxidoreductases to produce H_2, NADH and NADPH (Fig 6.1). As shown in table 6.3, hydrogen production from the different BG1 mutant strains is also very different. Strain BG1G1 grown on xylose produced the smallest amount of hydrogen and strain BG1G2 produced the highest amount of hydrogen.

In the presence of a functional LDH and expressed GLDH that can generate NADH, the lactate formation pathway could possibly be the preferred route over ethanol production pathway for the regeneration of NAD^+, and the extra reducing equivalents could be transferred to ferredoxin and released as hydrogen. However, when the lactate formation pathway was inactivated, the increased carbon flux was channelled toward acetyl-CoA and the cells adjusts its partitioning at the acetyl-CoA node to favour the production of ethanol to establish the redox balance under a more reduced environment. In this case, the additional NADH is oxidized via production of ethanol rather than H_2. According to Slapack *et al.*, the most important factors in regulating end-product ratios in thermophilic anaerobes are alcohol dehydrogenase, ferredoxin oxidoreductases and FDP-activated lactate dehdyrogenase. It is common that the $NADH/NAD^+$

cofactor pair has a regulatory effect on the expression of the genes and activity of the enzymes that influence the end-products distribution. Previous studies on *E. coli* also showed the introduction by NADH on the expression of the *adh*E gene that encodes the enzyme alcohol dehydrogenase [Leonardo *et al.* 1993, Leonardo *et al.* 1996], which catalyzes the production of ethanol during fermentation; and the inhibitory effect of high $NADH/NAD^+$ ratios on the pyruvate dehydrogenase complex [De Graef *et al.* 1999]. The activity of NAD^+ ferredoxin oxidoreductase in thermophilic anaerobic bacteria is strongly inhibited by NADH [Jungermann *et al.* 1973]. All these examples evidenced the regulatory effect of $NADH/NAD^+$ cofactor pair on the genes and the enzymes that could influence the end-products distribution.

Table 6.3 Comparison of genotype on metabolite molar yield using BG1 wild type, BG1L1, BG1G1 and BG1G2 strains.

	Glucose				Xylose			
Strain	Wild type	L1	G1	G2	Wild type	L1	G1	G2
$Y_{L/S}$ [(a)]	0.181	0.000	0.000	0.748	0.055	0.000	0.000	0.487
$Y_{A/S}$ [(b)]	0.082	0.083	0.082	0.128	0.134	0.098	0.042	0.175
$Y_{E/S}$ [(c)]	0.438	0.565	0.560	0.037	0.454	0.531	0.630	0.202
$Y_{H/S}$ [(d)]	0.088	0.126	0.131	0.226	0.077	0.121	0.037	0.223
Et/Ac [(e)]	5.353	6.808	6.863	0.285	3.384	5.406	14.86	1.155
CN (%)	94	97	96	99	94	95	101	105
[(f)]	(2.07)	(0.77)	(1.85)	(1.02)	(3.01)	(0.91)	(1.30)	(2.73)

Results are from anaerobic experiment using 5g/L of the carbon source supplemented with 2.5g/L glycerol in BA medium after 48 h of culture. Values shown are of triplicate cultures (a). Yield for lactate (Cmol/Cmol); (b). Yield for acetate (Cmol/Cmol); (c). Yield for ethanol (Cmol/Cmol); (d). Yield for hydrogen (Cmol/Cmol); (e). Carbon recovery; (f) Ethanol to acetate ratio

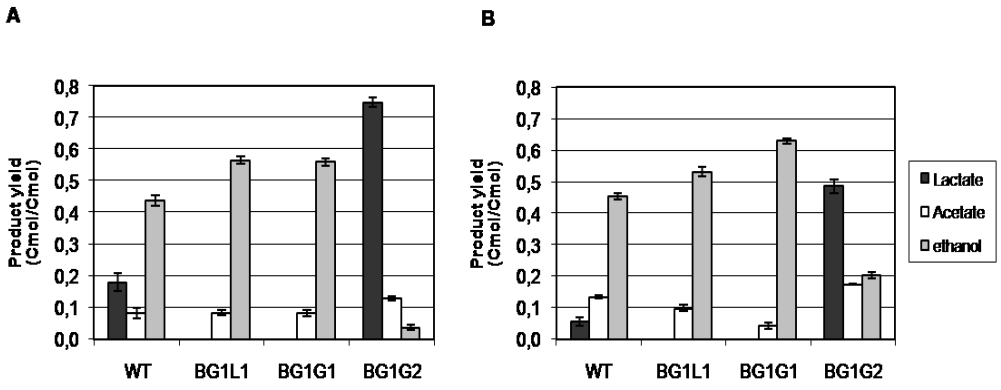

Figure 6.5 Products yield of BG1 wild type, BG1L1, BG1G1 and BG1G2 strains grown on glucose (A) or xylose (B) as substrate. Results are from anaerobic experiments using 5g/L glucose or xylose supplemented with 2.5g/L glycerol in BA medium after 48 h of culture. The values shown are for triplicate analyses and the error bars correspond to the SD for the triplicates.

6.5 Conclusions

This study demonstrates that it is possible to manipulate cofactor levels through metabolic engineering and therefore provides a way to study metabolite distribution in *T. mathranii* BG1 under the different cofactor levels. The use of mannitol as a carbon source increased ethanol production in BG1 wild type and *Δldh* strains, compared with the use of glucose and xylose. The result showed the importance of NADH as a rate-limiting factor in the ethanol formation pathway in strain BG1. The effect of the reducing power of mannitol can be observed by the dramastic increases in the ethanol-to-acetate ratio (Et/Ac). This altered Et/Ac ratio is a result of imbalanced carbon flux partitioning at the acetyl-CoA node, with more carbon flux directed toward the ethanol formation pathway.

To manipulate the cofactor level by overexpressing GLDH that can regenerate NADH, by eliminating the NADH competing pathway or by combining both strategies provoked significant changes in the metabolite distribution in *T. mathranii* BG1. With functional LDH and expressed GLDH, the lactate formation pathway could possibly be the preferred route over ethanol production pathway for the regeneration of NAD^+ and the extra reducing equivalents could be transferred to ferredoxin and released as hydrogen. With inactivated lactate formation pathway, the increased carbon flux was channelled toward acetyl-CoA and production of ethanol was favored to establish redox balance under a more reduced environment. In this case, the additional generated NADH is oxidized via production of ethanol rather than H_2. Therefore, the *ldh* negative strain with increased NADH availability caused by genetic manipulation should render *T. mathranii* BG1 industrially interesting for ethanol production.

6.6 References

Berrios-Rivera SJ, Bennett GN and San KY. 2002. Metabolic engineering of *Escherichia coli*: increase of NADH availability by overexpressing an $NAD^{(+)}$-dependent formate dehydrogenase. Metab Eng 4, 217-229.

Bryant MP. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. *Am J Clin Nutr*. 25(12):1324–1328.

De Graef MR, Alexeeva S, Snoep JL and Teixeira de Mattos MJ. 1999. The steady-state internal redox state (NADH/NAD) reflects the external redox state

and is correlated with catabolic adaptation in *Escherichia coli*. *J Bacteriol* 181, 2351-2357.

Georgieva T, Mikkelsen MJ and Ahring BK. 2007. Ethanol production from wet-exploded wheat straw hydrolysate by thermophili anaerobic bacterium *Thermoanaerobacter* BG1L1 in a continuous immobilized reactor. *Appl Biochem Biotechnol* 149:99-110

Hungate RE. 1969. A roll tube method for the cultivation of strict anaerobes. *Methods Microbiol* 3B, 117-132.

Jungermann K, Thauer RK, Leimenstoll G, Decker K. 1973. Function of reduced pyridine nucleotide-ferredoxin oxidoreductases in saccharolytic Clostridia. *Biochim Biophys Acta.* 305(2):268–280

Larsen L, Nielsen P and Ahring BK. 1997. *Thermoanaerobacter mathranii* sp. nov., an ethanol-producing, extremely thermophilic anaerobic bacterium from a hot spring in Iceland. *Arch. Microbiol.* 168, 114-119.

Lee SY and Papoutsakis ET.1999. ‘‘Metabolic Engineering’’ Dekker, New York.

Leonardo MR, Cunningham PR and Clark DP. 1993. Anaerobic regulation of the *adh*E gene, encoding the fermentative alcohol dehydrogenase of *Escherichia coli*. *J Bacteriol* 175, 870-878.

Leonardo MR, Dailly Y and Clark DP. 1996. Role of NAD in regulating the *adh*E gene of *Escherichia coli*. *J of Bacteriol* 178, 6013-6018.

Lopez de Felipe F, Kleerebezem M, de Vos WM and Hugenholtz J. 1998. Cofactor engineering: a novel approach to metabolic engineering in *Lactococcus lactis* by controlled expression of NADH oxidase. *J of Bacteriol* 180, 3804-3808.

Mikkelsen MJ, Ahring BK. 2007. World intellectual property organization, WO/2007/134607, *Thermoanaerobacter Mathranii* strain BG1

San KY, Bennett GN, Berrios-Rivera SJ, Vadali RV, Yang YT, Horton E, Rudolph FB, Sariyar B and Blackwood K. 2002. Metabolic engineering through cofactor manipulation and its effects on metabolic flux redistribution in *Escherichia coli. Metab Eng* 4, 182-192.

Slapack GE, Russel, I, Stewart GG. 1987. Thermophilic microbes in ethanol production. CRC, Boca Raton FL

Zeikus JG, Ben-Bassat A, Ng TK, and Lamed RJ. 1981. Thermophilic ethanol fermentations, In: Trends in the biology of fermentations for fuels and chemicals, Ed. A. Hollaender, Plenum Press, New York, 441

Appendix

List of Publications

A1: The results based on Chapter 2, 3 and 4 have been published in the following research article:

J Mol Microbiol Biotechnol 2010;19:123-133 (DOI: 10.1159/000321498)

Identification and Overexpression of a Bifunctional Aldehyde/Alcohol Dehydrogenase Responsible for Ethanol Production in *Thermoanaerobacter mathranii*

Shuo Yao[a], Marie Just Mikkelsen[b]

[a]BioScience and Technology Group, BioCentrum, Technical University of Denmark, Lyngby, and
[b]BioGasol ApS, Ballerup, Denmark

Shuo Yao
Biosystems Department, Risø National Laboratory for Sustainable Energy
Technical University of Denmark, Building 330, PO Box 49, Frederiksborgvej 399
DK–4000 Roskilde (Denmark)
Tel. +45 4677 4125, Fax +45 4677 4109, E-Mail shya@risoe.dtu.dk

A1: The results based on Chapter 5 and 6 have been published in the following research article:

Appl Microbiol Biotechnol (2010) 88:199–208
DOI 10.1007/s00253-010-2703-3

APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Metabolic engineering to improve ethanol production in *Thermoanaerobacter mathranii*

Shuo Yao · Marie Just Mikkelsen

S. Yao
BioScience and Technology Group, BioCentrum,
Technical University of Denmark,
2800 Lyngby, Denmark

M. J. Mikkelsen
BioGasol ApS,
Lautrupvang 2A,
2750 Ballerup, Denmark

S. Yao (✉)
Biosystems Department,
Risø National Laboratory for Sustainable Energy,
Technical University of Denmark,
Building 330, P.O.B. 49, Frederiksborgvej 399,
4000 Roskilde, Denmark
e-mail: shya@risoe.dtu.dk

Risø DTU is the National Laboratory for Sustainable Energy. Our research focuses on development of energy technologies and systems with minimal effect on climate, and contributes to innovation, education and policy. Risø has large experimental facilities and interdisciplinary research environments, and includes the national centre for nuclear technologies.

Risø DTU
National Laboratory for Sustainable Energy
Technical University of Denmark

Frederiksborgvej 399
PO Box 49
DK-4000 Roskilde
Denmark
Phone +45 4677 4677
Fax +45 4677 5688

www.risoe.dtu.dk